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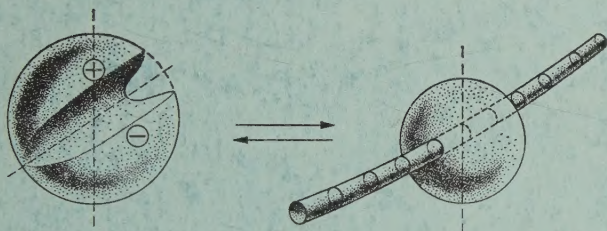
CONFORMATIONAL CHANGES IN ENZYMES

ACCOMPANYING CATALYSIS

BY

B. H. Havsteen

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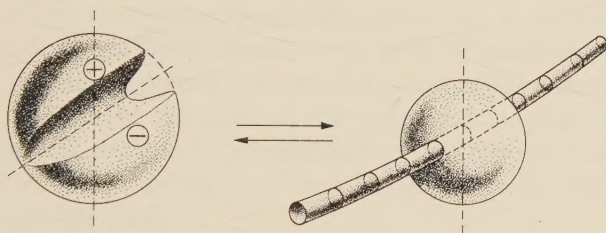
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B. H. Havsteen



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1968

Denne afhandling er af det matematisk-naturvidenskabelige fakultet
ved Københavns universitet antaget til offentligt at
forsvares for den filosofiske doktorgrad.

København, den 26. marts 1968.

MORTEN LANGE

h. a. dec.

Preface

The experimental work reported in this dissertation was performed in the period of 1962–66 in the Department of Biochemistry of Cornell University in Ithaca, New York (1962–63) and at the Max-Planck-Institut für physikalische Chemie in Göttingen (1963–66).

I wish to express my sincere gratitude to Professor George P. Hess and to Professor, Dr. Dr. h.c.mult. Manfred Eigen, the former for introducing me to the methods of investigation of the structure of proteins and to mechanism of their biological action, the latter for teaching me the theory and application of the methods of chemical relaxation. Both provided me excellent working conditions. Director, Dr. L. DeMaeyer of the Max-Planck-Institut für physikalische Chemie greatly helped me with the technological problems in connection with the construction of the flow-temperature-jump apparatus. I am also indebted to Professor H. A. Scheraga for the permission to work in his laboratory over several years at the spectropolarimeter and for his considerable contribution to my education in the field of the physical chemistry of biological polymers. I am grateful to Professor, dr. med. F. Schønheyder of the Institute of Biochemistry at the University of Aarhus for the time I was allowed to use for the editorial work in connection with the thesis.

The financial support from NATO (Science Fellowship, 1964–65) Danmarks Almindelige Videnskabsfond (fellowship, 1964–65) and the Max-Planck-Institut für physikalische Chemie (1965–66) is gratefully acknowledged.

Finally, I wish to thank my relatives and friends for their patience and consideration while I was occupied by this work.

August, 1968.

BENT H. HAVSTEEN

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Abbreviations

A_{280}	Light Absorbancy at 280 nm.
a_0, b_0	Optical rotatory dispersion parameters.
$[\alpha]$	Specific rotation.
AcCT	Monoacetyl- α -chymotrypsin.
Ac-L-val-OMe	N-acetyl-L-valine methyl ester.
AF	Acriflavine, 10 methyl-3,6-diamino-acridine.
AO	Acridine orange, 3,6-di (dimethyl)-amino-acridine.
ATEE	N-acetyl-L-tyrosine ethyl ester.
ato	Atmospheres above normal pressure.
$\bar{c}$	Equilibrium concentration.
CT	α -Chymotrypsin.
Cin-CT	Cinnamoyl-chymotrypsin.
CTgen	Chymotrypsinogen.
DACCT	Deacylated AcCT.
DFP	Diisopropyl-fluorophosphate.
DIP-CT	Diisopropyl-phosphoryl-chymotrypsin.
Δ	Difference.
E	Enzyme.
ϵ	Molar, decadic extinction coefficient.
DEP	Diethyl-propyl-.
EDTA	Ethylene diamine tetraacetic acid.
e.u.	Entropy units.
ES and ES'	Enzyme-substrate complexes.
FCN	Cyanoferrate.
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase.
GU	5 M guanidinium hydrochloride, 1.2 M urea.
G3P	Glyceraldehyde-3-phosphate.
I	Inhibitor.
ibid.	In the same book, journal, etc.
k_R	Rate constant for recombination.
k_D	Rate constant for dissociation.
$k\Omega$	Kilo ohm.
K_a	Acid dissociation constant.
K_i	Inhibition constant.
K_M	Michaelis' constant.
K_{spec}	Spectrophotometrically determined equilibrium constant.
K_{stab}	Stability constant.
λ	Wavelength.
M	Moles per liter.
μ	Ionic strength.
Mc	Mega cycles.
m.r.	Molar ratio.
met	Methionine.
M_0	Mean residue weight.
$M\Omega$	Mega ohm.
n	Refractive index.
N	Normality, equivalents per liter.

N	Noise.
NAD	Nicotin adenine dinucleotide (NADH: Reduced NAD).
nm	Nanometer ($m\mu$).
$n-\pi^*$	Electronic transition from the non-bonded (n) orbital to the antibonding orbital (π^*).
N-Ac-3,5-dinitro-L-tyr	N-Acetyl-3,5-dinitro-L-tyrosine.
O.R.D.	Optical rotatory dispersion.
P	Reaction product.
p-	Para-.
$Q^{\cdot-}$	p-Benzoquinon semiradical ion.
[Q]	Molarity of p-benzoquinon.
$[QH_2]$ or $[Q^=]$	Molarity of hydroquinon.
τ	Relaxation time.
Pt	Platinum.
PF	Proflavine, 3,6-diamino-acridine.
3-PGA	3-Phosphoglycerate.
S	Substrate model; or substrate.
S	Signal.
s or sec.	Second.
S_y	Standard error about the ordinate.
T	Temperature.
T-jump	Temperature-jump.
Tr	Trypsin.
Tris	Tris-(hydroxymethyl)-aminomethane.
UV	Ultraviolet.
V_{max}	Maximal velocity in the terminology of Michaelis.
vs	Versus.
% v/v	Volume percent.
w	Electrostatic factor.
Å	Ångström units = 10^{-8} cm.
>	More than.
<	Less than.
$\approx$	Almost equal to.
∞	Corresponding to.
$-o\rightarrow$	Isomerization.

Chapter 1

Introduction

The mechanism of enzyme catalysis has been the subject of many investigations¹. There early was a general agreement about considering this process a series of many consecutive steps, which possibly also had some parallel pathways. However, in all these steps the enzyme was expected to play the classical role as a rigid particle with a fixed active site (the “Lock and Key” theory of Emil Fischer and the template theory)². It was not until about 1960, that the idea became widespread that the enzyme, at least in some cases, was a flexible molecule which underwent structural rearrangements in the course of the catalytic process.

The protein chemistry was then so advanced that it was clear that the molecular forces which maintain the three-dimensional structure were relatively weak and delicately balanced. Therefore, small external perturbations could often release isomerizations in the polypeptide structure. This resulted in changes in the physical and biological properties of the macromolecule. Such structural changes were often reversible, at least after suitable tempering processes, provided that all covalent linkages remained intact or were restored after the perturbation³.

The best known of the configurational changes in biopolymers are the unfolding reactions known as denaturation. This phenomenon is by definition accompanied by a loss of biological function. Proteins can, however, also fold to a tighter structure following a perturbation such as the binding of a small molecule or ion^{4, 5}. Familiar examples of that are substrate binding to enzymes and antibody-antigen interactions. The nature of the forces involved is not entirely unravelled, but there is no doubt that charge neutralization and interphase phenomena such as surface tension play a important role.

Theoretical considerations have predicted⁶ and direct observations by x-ray diffraction analysis have confirmed^{7, 49} that the polar sidechains are located in contact with water on the surface of the macromolecule whereas the non-polar moieties normally are rather tightly packed in the internal of the polymer. The effect of the hydrophobic parts of the protein on the surface tension favours the organization of water aggregates near the polypeptide surface. In turn, these crystalline water structures (ice) lend considerable stability to the macromolecule. These are the so-called hydrophobic interactions. Such interactions consist of a number of singly non-specific contributions to the free energy, but taken as a

whole they provide highly specific surfaces of contact between various parts of the protein^{6,7}.

It may be shown that changes in the polarity of the solvent, e.g. by addition of an organic component^{8,9}, disturbs the hydrophobic interactions and in turn causes alterations in the spectrum of the protein in the ultraviolet range. The latter is a sensitive indicator of the conformation^{1,10}. Similarly, one must expect that addition of a non-polar substrate or an inhibitor to an enzyme results in rearrangements as well in the cage of crystalline water around the amino acid sidechains as in the polypeptide structure proper to minimize the free energy of the complex. Such effects have been observed⁸. The binding of non-specific, non-polar compounds only gives rise to small, trivial rearrangements in the enzyme, whereas specific interaction can release drastic changes in its conformation and the associated biological properties.

There are now many pieces of direct and indirect evidence of the occurrence of conformational changes in enzymes after specific binding of substrate or inhibitor (see p. 15). It also seems that most workers in the field now accept that the theory is valid. However, in the question of the significance of this phenomenon to the mechanism of enzyme action, there are differences of opinion. The problem has two important aspects: Specificity and catalysis. Each of these properties must be regarded from a steric and a thermodynamic point of view. Whichever prevails of these effects must be decided by experimentation in each case. The definite juxtaposition of the essential participants during catalysis or the opening and closing of the approach to the active site may be the basis for a geometric control of the catalytic efficiency by the conformational change. The thermodynamic importance of the structural isomerizations may be a favourable energy transformation from structural (i.e. potential) energy to a form of free energy which is capable of lowering the activation barrier for the substrate conversion. The latter idea is at present largely speculative, but appears to be plausible.

A newly discovered, special kind of conformational changes in enzymes, caused by binding of a small, specific compound, are the allosteric effects. The concept was largely developed by Wyman, Monod and coworkers¹¹. The term covers the phenomenon that enzymes of this type can exist as two conformational isomers which are in mutual equilibrium, possess individual catalytic properties and are capable of binding a ligand. The allosteric isomers of the enzyme generally, but not necessarily, bind the ligand with different affinity¹¹. The ligand, which usually is not the normal, specific substrate of the enzyme shifts the balance between the two enzyme isomers by the mass law effect. Thus, the catalytic efficiency and the direction of the reaction path is controlled.

This hypothesis provides the only known scheme which adequately explains the kinetic and static data for many enzymes consisting of an even number of identical subunits. The curves for the binding of the ligands (so-called "allosteric effectors") to an allosteric enzyme and the dependence of the $V_{\max}$ -parameter of Michaelis on the concentration of this ligand have a characteristic, sigmoidal

shape instead of the usual, hyperbolic relationship. The inventors¹¹ of the allosteric hypothesis postulate that an allosteric enzyme always consists of an even number of identical subunits which are arranged in a symmetric fashion and that the symmetry is preserved during the isomerization. So far, several enzymes displaying "allosteric" effects have indeed been shown to consist of an even number of at least almost identical subunits, but the symmetry requirement is seriously in question.

The allosteric hypothesis is now supported by considerable kinetic evidence for yeast glyceraldehyde-3-phosphate dehydrogenase, where the ligand is NAD¹². In the cases of the binding of cytidine triphosphate to aspartate transcarbamylase¹³ and of oxygen binding to haemoglobin¹⁴, the experimental data are also quite convincing. However, it is yet premature to maintain, that the model generally is applicable. If this is true, however, then the phenomenon of allosterism is likely to be of great importance to the control of metabolic pathways.

Chapter 2

Review of the literature on conformational changes accompanying enzyme catalysis

The hypothesis that conformational changes in enzymes play an important role in their catalytic function was originally proposed in various forms by von Euler and Josephsen (polyaffinity)^{15, 16} and by Eyring, Lumry and Spikes¹⁷. The original idea was that the enzyme acted upon the substrate like the mediaeval instrument, the rack¹⁷. The authors imagined that the substrate was suspended between two or more sites on the enzyme. The points of attachment were then moved apart until the weakest linkage in the substrate ruptured.

Adequate experimental evidence of the occurrence of conformational changes in enzymes during their catalytic activity could not appear until the modern methods of purification and characterization of proteins had been developed. An extensive presentation of these methods would exceed the frames of this dissertation. It is presented elsewhere^{1, 10} and must, as well as a knowledge of thermodynamics and protein structure in general, be assumed to be known to the reader. Here, it must be sufficient to mention a number of the more important techniques. They include measurement of volume and energy of activation, difference spectra, optical rotatory dispersion, enthalpy and entropy of denaturation, protolytic titration curves, enthalpy of ionization, sedimentation and diffusion coefficients. Furthermore, methods such as light scattering, amino acid analysis, x-ray diffraction, chemical modification by esterification, alkylation, amidination, selective oxidation, counting of ionizing radiation and determination of rate constants by flow, stopped-flow, quenched flow, and chemical relaxation methods are important. It is hoped that these names, together with structural terms, such as active sites, binding sites, essential amino acid residues, stability, flexibility and names of enzymes, such as chymotrypsin, trypsin, ribonuclease, myoglobin, lysozyme and miramidase, may act as "light houses" for the "navigation" through the mass of evidence and examples in the literature.

The account of the previous work is chronological whenever possible because this appears to be the most logical way to present the evolution and gradual establishment of the theory of conformational changes in enzymes accompanying their catalytic action. It may appear appealing to divide the material according to the method of investigation. The clarity which could be obtained in this way

would, however, be outweighed by a necessity for frequent repetition of the same work because the same authors usually employ a considerable number of independent techniques to check their results.

Smith considered the possibility of the occurrence of informational changes in the enzyme when he proposed his theory for the action of metallopeptidases¹⁸. Additional evidence was found for luciferase: The volume of activation measured was very large. This observation was difficult to interpret without assuming a change in the structure of the enzyme since the substrate is a small molecule¹⁹. Laidler²⁰ contributed by demonstrating the occurrence of unusually large changes in entropy and volume during the slow step of the catalysis by acetylcholinesterase and urease.

Vaslow and Doherty^{21,22} discovered that the binding of many substrates to chymotrypsin was characterized by a very low entropy change (0–38 e.u.). They concluded that the binding process induced a conformational change, and specifically a helix formation, in the enzyme. However, Scheraga, Némethy and Steinberg²³ have pointed to an alternative explanation. They have shown that the entropy of formation of hydrophobic interactions between sidechains can have large negative values (8.6 e.u. at 25° C for a single leucine-isoleucine interaction). Since it was then unknown how many hydrophobic interactions were disturbed by the substrate binding the question of the conformational change remained open. Also Linderstrøm-Lang and Schellman²⁴ examined the data on the entropy change and coulombic interactions at the binding the substrate to the enzyme. They had to conclude, that much still remained to be clarified before the thermodynamics of this process could be considered to be established.

An important proposal was made by Koshland and Thoma²⁵ who suggested that the substrates of β -amylase induce conformational changes in the enzyme as they approach the binding site. This hypothesis, which is called the "induced fit" hypothesis, proposes that the active site is formed during the binding process and is eliminated as the products leave the enzyme. It resembles the scheme outlined in this work (see chapter 5) in postulating the occurrence of conformational changes during its interaction with a specific substrate, but it differs from this thesis because it only mentions structural isomerizations in the enzyme in connection with the binding process.

The name of the hypothesis is a lucid description of the phenomenon, but the term "induction" should probably not be taken literally as a sort of long distance field effect in direct analogy to electromagnetic induction. It should sooner be regarded as a simple bimolecular binding process which is followed by an isomerization of the enzyme-substrate complex.

Moon and Hess^{26,27} obtained some rather direct evidence of the occurrence of conformational changes in chymotryptic catalysis. They found that the rate of liberation of p-nitrophenol from its acetyl ester could be rate determining although it was expected to occur rapidly. The same was found for the release of HF in the catalytic hydrolysis of DFP by chymotrypsin.

The discovery of Cohen and Erlanger²⁸, that diethyl-phosphoryl-chymotrypsin is more stable than α -chymotrypsin at 50° C and pH 7, as measured by the specific activity after reactivation of the former compound with a nucleophile, was attributed to a conformational change. In the light of other evidence, this appeared to be correct but caution seems necessary until it is possible to exclude some alternative explanations: Steric hindrance by the DEP-group or autolysis of chymotrypsin.

Szabolcsi and Biszku²⁹ found that aldolase, which had been subjected to slight denaturation by modification of some of the sulfhydryl groups, was more susceptible to limited proteolysis than the native enzyme. Since addition of substrate to the denatured enzyme restored the resistance towards limited proteolysis, it was concluded that the substrate had induced a conformational change in the enzyme. This was supported by the failure of the sulfhydryl groups to appear in a titrable form after the formation of the enzyme-substrate complex.

Kunitz and McDonald³⁰ early noted that many enzymes were stabilized against inactivation by thermal unfolding by specific substrates and inhibitors. This supports the concept of a change in the enzyme conformation during the catalytic process to a denser and chemically more resistant structure.

One of the important pieces of evidence of the ability of a substrate to release a conformational change in an enzyme was found by Anfinsen⁵. He and his collaborators discovered that ribonuclease which had been fully unfolded rapidly refolded to the native, active configuration after addition of a specific substrate, such as uridine-3-phosphate. Similar evidence of the protective effect of specific substrates on ribonuclease was later reported by Barnard³¹. He found that phosphate stabilizes ribonuclease towards unfolding by eight molar urea and induces a refolding reaction in the denatured enzyme. The efficiency in this reaction of a series of phosphates depended on their binding affinity.

Evidence of the thesis from chemically modified enzymes has provided some useful information. Citri and Garber³² found changes in the reactivity of penicillinase towards an iodinating reagent upon addition of a competitive inhibitor. This is probably due to a substrate-induced conformational change. It seems, however, necessary to consider simple steric hindrance a possible alternative explanation.

The optical rotation of a macromolecule, such as a protein, is a very sensitive indicator of conformational changes. Experimental evidence based on polarimetric measurements therefore early appeared in the literature. Neurath, Rupley and Dreyer³³ reported that diisopropyl-phosphoryl-chymotrypsin, an analog of an intermediate in the chymotryptic catalysis of specific substrates, had a less negative specific rotation than the free enzyme or the precursor, chymotrypsinogen. This indicated that a conformational change occurred in an early phase of the catalytic process, because the actual catalytic step took place after the stage represented by the trapped intermediate, DIP-CT. This work initiated many supplementary investigations by Lumry and Parker³⁴ and by Hess and his co-

workers^{4,46}. Similar evidence soon appeared for creatine kinase³⁵. Thus, the phenomenon did not seem to be confined to hydrolases. This finding greatly increased the interest in the phenomenon because, if isomerization during catalysis was a general property of enzymes, rather than a peculiarity of a restricted group of catalytic proteins, then the conformational changes would have to be taken into consideration whenever hypotheses for the mechanism of enzyme catalyses were developed. The view, that the conformational changes in enzymes are important elements of their catalysis, has gained wide acceptance in the field over the years and is held by the large majority of the leading specialists in enzyme action.

Samuels, Nihei and Noda³⁵ observed a large change in optical rotation and its wavelength dispersion upon addition of adenosine diphosphate, the specific substrate, creatine phosphate, and a magnesium salt to creatine kinase. No changes in optical rotation were observed, unless all the four components were present and the direction of the effect was opposite to that caused by urea denaturation of the enzyme. Similar, but much more drastic effects have been found by Yagi and Ozawa for D-amino acid oxidase³⁶⁻³⁹, an enzyme which requires flavine adenine dinucleotide as a cofactor.

Wootton and Hess⁴⁰ discovered the difference spectrum between DIP-chymotrypsin. They found the identical difference spectrum between monoacetyl- α -chymotrypsin, AcCT, and the free enzyme. This difference spectrum therefore seemed to be a general property of specifically acylated chymotrypsin. AcCT is a true, kinetic intermediate in the catalytic reaction. It may be isolated at pH-values (pH 2-6) which are somewhat lower than the range corresponding to maximal activity. A careful characterization of the difference spectrum, which is located as 270-300 nm, showed that some of the aromatic sidechains of chymotrypsin were involved: About one half of a tryptophyl residue in DIP-chymotrypsin were protected against specific oxidation by N-bromosuccinimide, whereas all the seven indolyl groups could be oxidized in α -chymotrypsin. Williams and Laskowski⁴¹ supported this finding by demonstrating that a fraction of a tryptophyl residue is protected against solvent perturbation (by glycerol, ethyleneglycol or polyethyleglycol) in DIP-chymotrypsin, while it is exposed in α -chymotrypsin. Wootton and Hess were able to conclude that covalent substitution and charge effects were not involved and postulated that an indolyl chromophore was the origin of the difference spectrum.

Oppenheimer, Mercouroff and Hess⁴² were able to confirm the hypothesis very convincingly with a model system. They measured the difference spectrum between indole in benzene and indole in water and found virtually the identical spectrum to that between any of the isolated chymotrypsin-substrate intermediates and the free enzyme. Hence, the environment of an indol sidechain in CT had become less polar during one of the first steps following substrate binding to the enzyme.

Biltonen and coworkers⁴³ carried the analysis a step further by the use of optical rotatory dispersion (O.R.D.) measurements in the far ultraviolet range. They found, that the O.R.D. changes associated with the arising of difference spectrum at 290 nm, were due to the tail end of a Cotton effect near 225 nm.

At this stage in the development, a need was felt for a critical review of the large number of experiments reported on the subject and of the many hypotheses forwarded. This task was undertaken by Bruice⁴⁴ who was able to revise and eliminate many of the early proposals and arrive at a clear picture of the situation. Since then, the proliferation of articles in the field has continued. Here, the review of this period will be restricted to the papers which appear to be the most important.

Platt and Niemann⁴⁵ reported that the rate constant for chymotryptic catalysis of the hydrolysis of substrates for which the enzyme is specific, such as nitrophenyl acetate or DFP, is pH-dependent in the pH-range of high enzymatic activity. Since this observation was supported by the discovery of Hess et al. of a concomitant pH-dependent change in the proton balance on the enzyme and a change in the O.R.D.-parameters in the same pH-range, it was concluded that the active site of α -chymotrypsin rearranged in the course of the catalytic process⁴.

Labouesse, Oppenheimer and Hess⁴⁶ corroborated this conclusion by their work on a derivative of α -chymotrypsin which was prepared by normal activation of chymotrypsinogen in which all the ϵ -amino-groups had been masked by acetylation. These authors found by polarimetric measurement that the conformation of the acetylated chymotrypsin (with free N-terminal ends of the B and C-chains) was determined by the charge on the α -aminogroups and that only the ammonium form had catalytic activity. The acetylated chymotrypsin was almost fully active in a pH-range of maximal activity of the native enzyme, but all activity was lost upon reacetylation which masked the N-terminus. A brief exposure to an alkaline pH hydrolyzed the acetyl group on the α -amino group of the B-chain (iso-leucine) and restored almost full activity. This important result identified the α -aminogroup of the B-chain N-terminus as an essential participant in the catalysis by chymotrypsin and gave an indication of the role of this group in the preparatory phase of the hydrolysis: Control of the equilibrium between the active and the inactive configuration or of the port of entry for the substrate to the catalytic site.

One of the most direct and unambiguous ways of detecting changes in molecular structure in general, and conformational isomerizations in particular, is x-ray diffraction. Fortunately, this approach is feasible for identification of substrate induced rearrangements in enzymes. Often, the movements are of the order of a few Ångström, i.e. so small that the required optical resolution is very high. Therefore, a final assignment of a position to every atom in the molecule is sometimes only possible when it has been confirmed by a chemical method, such as bridge formation between sidechains which sterically are closely located, but are widely separated in the sequence of the main chain.

Sigler and Skinner⁴⁷ studied the changes in the x-ray diffraction pattern of α -chymotrypsin which occurred after addition of DFP. Similar studies were made by Blow et al.⁴⁸ on α -chymotrypsin. Both groups found small but definite changes in intensity and position of the diffraction pattern and a small distortion of the lattice (the unit cell). The movements of the chains therefore appear to be quite small and localized. Yet, they are of great importance to the catalytic function.

The work of Phillips⁴⁹ on lysozyme includes x-ray experiments which irrevocably show that the active site and other parts of the enzyme have different conformations in the various phases of the binding and the substrate cleavage steps. The fitting of models to the diffraction pattern even permit the assignment of individual groups to each single step and each function in the mechanism.

Also Richards⁵⁰ found such changes in the x-ray diffraction pattern of an enzyme when it was brought in contact with a substrate. In this case, a crystal of ribonuclease was mounted in a tube through which a solution of substrate passed. The displacement of the diffraction spots and the change in their intensity after the substrate had reached the enzyme crystal were quite marked. A difficulty in such experiments is that special attention has to be given to the flow conditions. Otherwise, the effect may be controlled by diffusion rather than by the catalytic reaction and the molecular change measured may be smaller than it actually is under physiological conditions.

The question has been raised by Kendrew⁵¹, whether or not the x-ray data from proteins in the crystalline state may be directly transferred to the structure of the dissolved protein, i.e. whether the protein has the same conformation in the crystalline state as in solution. If this is not the case, then the value of the x-ray work, which practically is confined to the solid phase, is considerably diminished. Almost all x-ray crystallographic measurements on proteins are therefore now made on crystals wetted with a saturated solution of the macromolecule. This precaution should minimize the error due to conformational changes accompanying dissolution (changes in hydration of the protein upon dissolution). The question may be investigated in the case of myoglobin⁵¹, where it is possible to compare estimates of the helical content from optical measurements in the UV range (O.R.D., circular dichroism etc.) with the actual helicity directly observed on the model which fits the diffraction data of Kendrew⁵¹. The agreement was good, but that is not necessarily the case in general, as the stability of proteins vary considerably. Praissman and Rupley⁵² investigated this problem using the rate of tritium-hydrogen exchange in crystal suspensions and in the corresponding solutions. They found the same exchange rate of tritium in the crystalline as in the dissolved lysozyme.

An example, of how chemical experiments can aid the detailed structural investigation by x-ray diffraction analysis, was given by Schachter et al⁵³. They oxidized the methionine residues in chymotrypsin (CT) and trypsin by hydrogen peroxide in the presence and absence of a specific substrate. In the case of CT, they chose N-acetyl-L-tyrosine ethyl ester as the substrate. Both of the methionine

residues (met) in chymotrypsin (met-180 and met-192 according to the amino acid sequence of Blow and coworkers⁵⁴) were oxidized by H_2O_2 in the presence of acetyl-tyrosine ethyl ester, whereas only met-192 (besides one cysteine and 1–2 tryptophyl residues) was destroyed in the absence of the specific substrate. The remaining methionine residue therefore seems to be either directly covered by the substrate or protected by a fold of the peptide chain, which is located elsewhere in the native enzyme. Oxidation of met-180 results in a drastic loss in biological activity of chymotrypsin while destruction of met-192 only slightly affects the catalytic power. The former residue therefore appears to participate in the enzymatic action, presumably by stabilizing the conformation of the active site. Since the binding and the catalytic sites are believed to be remote from the methionine residue, which is protected by the ester, a likely explanation of the phenomenon is, that the masking effect is due to substrate induced conformational changes which bring a peptide chain into the vicinity of met-180.

Also deuterium-hydrogen exchange experiments have been used to test the possibility of occurrence of enzyme rearrangement in an enzyme induced by substrate and cofactors (Hvidt, Kägi and Ottesen^{55,56}). These authors measured the infrared absorption of the so-called amide II band of the yeast alcohol dehydrogenase apoenzyme in the presence and absence of NAD, NADH and ethanol. Marked alterations in the rate of this exchange reaction were found on addition of the small, specific molecules to the protein part of the enzyme.

The direct observation by Phillips⁴⁹ of a conformational change in lysozyme (from egg white) following the binding of a competitive inhibitor was corroborated by Hayashi, Imoto and Funatsu⁵⁷, who worked with muramidase which is a bacterial variety of the enzyme. A difference spectrum with absorption maximum at 253.5 nm arose when the enzyme was allowed to react with its specific substrate, chitin, but not when the enzyme was substituted by a mixture of tryptophane and tyrosine or by albumin. The difference appears to arise from a tryptophyl residue in the enzyme, since specific oxidation of indole with N-bromosuccinimide reduces the differential extinction coefficient in proportion to the disappearance of the indole group. In this respect and in the inactivation of the enzyme, which parallels the oxidation of the first tryptophane residue in the enzyme, muramidase resembles chymotrypsin. The presence of the substrate produces a shift towards longer wavelengths in the UV-spectrum of a unique tryptophyl residue in muramidase. This finding is compatible with the occurrence of a change in position of the indole sidechain, i.e. a substrate induced conformational change, but could also be due to direct perturbation by a part of the polymeric substrate.

The work of Hess and coworkers⁵⁸ (see also 3.1.1.) showed that the conformational changes in chymotrypsin were accompanied by a very large shift in the dissociation constant of the α -amino group of the isoleucyl residue which forms the N-terminus of the B-chain. This charge difference at normal pH-values and the change in symmetry (shape) of the molecule found by these authors was the

basis for a comparative study by Nakamura, Takeo and Sasaki⁵⁹ of the electrophoretic mobility of a number of enzymes in the presence and absence of specific substrates or inhibitors. During the ionophoresis of the enzyme, the substrate or an inhibitor was allowed to migrate by gravity and capillary forces in a direction perpendicular to the movement of the enzyme. In the cases of chymotrypsin and trypsin, a change in the direction of the enzyme migration was seen when the substrate analogue, DFP, reached the enzyme. This was, however, not the case when these enzymes were substituted by their precursors. This result is an independent confirmation of the optical and chemical work of Hess⁴⁶.

The polarimetric work on the conformational changes of chymotrypsin by Hess and the author⁸ (see also p. 26) and by Lumry and Parker³⁴ was repeated by Weiner and Koshland⁶⁰ using a continuously recording spectropolarimeter capable of penetrating deeper into the ultraviolet range than it was possible with the older, hand-operated equipment. Their data could only confirm the early findings qualitatively because the experimental conditions were not identical with our, but Weiner and Koshland arrived at the same conclusion as we did: The observations indicated the occurrence of conformational changes in chymotrypsin following the binding of specific substrates or inhibitors.

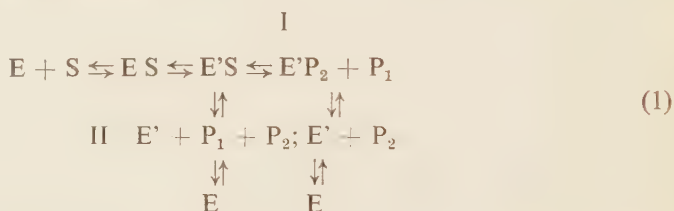
Lumry and coworkers⁴³ continued their extensive optical and thermodynamic investigations of the chymotrypsin-substrate and chymotrypsin-inhibitor systems and found further evidence of the correctness of the proposed hypothesis. Its theoretical basis was expanded by Hammes⁶¹. He postulated that entropic compensation effects in an enzyme, which is undergoing conformational changes in response to substrate binding, can lower the energy of activation for the substrate reaction.

Gutfreund and coworkers⁶² were the first to make use of a stopped-flow technique for detection of conformational changes accompanying catalysis. They studied the specific catalysis by chymotrypsin and trypsin. The reaction was initiated by mixing the outflow of two syringes, one containing enzyme solution, and the other the substrate, in a T-tube. The extent of the reaction was detected by following the change (optical density measured in the outflow from the mixing chamber) in the spectrum of a chromophoric, competitive inhibitor, proflavine, upon binding of the substrate. The enzyme was charged with proflavine before the substrate was added. In the course of the reaction of enzyme with substrate, the dye was displaced thus eliminating the perturbation of the chromophore by the enzyme. A slow isomerization was found to precede the catalytic steps. This is in agreement with the findings of Moon, Sturtevant and Hess²⁶ who used diisopropyl-fluorophosphate as a substrate model.

Gutfreund and collaborators⁶³ confirmed their results from the dye displacement experiments by use of chromophoric substrates, such as *N*- β -(2-furyl)-acroleyl-L-tyrosine ethyl ester. This substrate has the advantage that the moiety near the bond to be cleaved carries the typical specificity of chymotrypsin and that the chromophore is not in conjugation with the carbonyl group of the ester or amide

linkage. Therefore, a possible marked electronic influence of the chromophore on the critical substrate bond is eliminated. Such a criticism is more valid in the case of cinnamoyl-imidazole, a substrate which often is used⁶⁵.

Gutfreund and coworkers⁶⁴ also introduced the stopped-flow quenching method in the study of kinetically important conformational changes. They mixed a solution of the enzymes with one of substrate in an efficient mixing chamber and led the reaction mixture through a thin plastic tube to a vessel which contained trichloroacetic acid. The latter arrested the reaction within a few milliseconds by inactivation and precipitation of the enzyme. Gutfreund and his collaborators were able to show that the alcohol was liberated at a much slower rate than the acid. This finding is incompatible with the classical acyl-enzyme mechanism for hydrolases^{65,66}, but it fits a scheme where the rearranged enzyme-substrate complex (E'S, see the scheme below) forms a branch point from which the reaction can proceed along alternate pathways:



If S is an ester, then P₁ is the liberated alcohol and P₂ the acid. The nature of the leaving group (P₁) of the ester has an important influence on which of the alternate pathways is prevailing. Substrates with labile leaving groups, such as phenol derivatives, follow the acyl-enzyme scheme (I), whereas the real (amide and ester) substrates seem to be decomposed along both path I and II simultaneously.

It appears that not only enzymes, but also other catalytic proteins, such as the cytochromes in respiratory chain undergo important conformational changes in the course of their biological function, facilitation of the electron transfer to the ultimate acceptor. Greenwood⁶⁷ found by stopped-flow studies of the interaction of this pigment with various redox couples, that the kinetics and the pH-dependence of the rate parameters only could be interpreted in terms of an isomerization of ferricytochrome c preceding the change in oxidation number. Hess and Czerlinski⁶⁸ arrived at the same conclusion from kinetic investigations. They used as well the relaxation of a transient perturbation (the temperature-jump technique) as the stopped-flow approach. Thus, the view, that conformational changes in macromolecules accompany their biological action, has become widespread.

2.1. WORK DIRECTLY PRECURSORY TO THE DISSERTATION

The acquisition of a profound insight in an unknown, intricate mechanism, such as that of enzyme catalysis, requires a thorough knowledge of many experimental

results which have been accumulated over the years. These findings must be critically examined and coordinated. An attempt to do this has been made in the first part of the thesis. This formulation of status quo formed the basis for the research project presented here. Since a major part of this dissertation deals with chymotrypsin and since the experiments reported here grew out of the

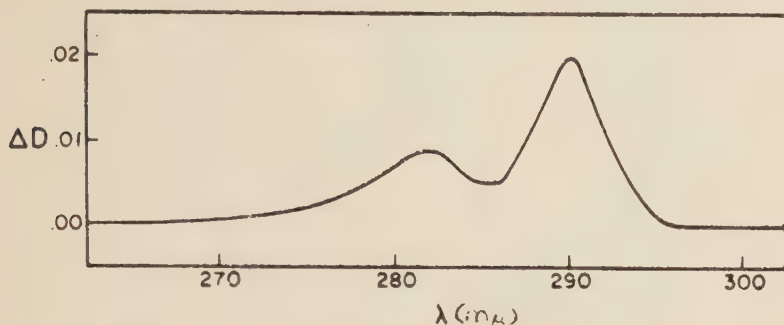


Figure 1

Ultraviolet difference spectrum, DIP-CT versus CT, pH 6.7 (0.033 *M* Tris (hydroxymethyl)-aminomethane-HCl buffer, 0.033 *M* in CaCl_2), 3.6×10^{-5} *M* enzyme, 1.1×10^{-4} *M* DFP 25°. Scanned 3 minutes after addition of DFP to sample cell of a Cary Model 14 self-recording spectrophotometer.

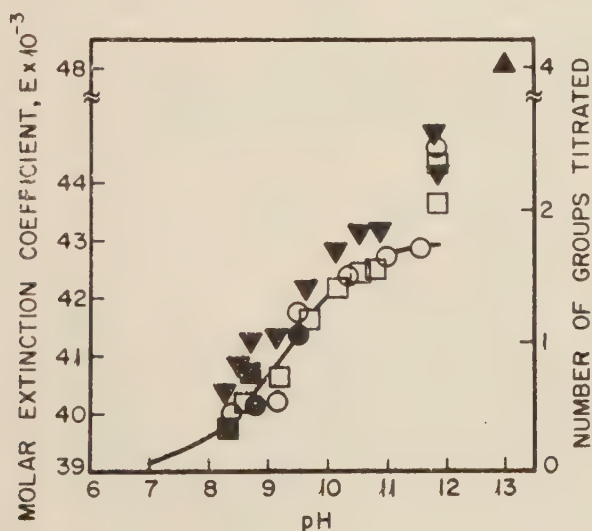


Figure 2

Spectrophotometric titration curves of CT and DIP-CT in 0.70 *M* KCl at 16° at a wavelength of 290 nm. The open circles and squares are for the forward titrations of CT and DIP-CT, respectively, the filled circles and squares are for the corresponding back titrations. The inverted triangles are for the forward titration of DIP-CT, uncorrected for the 290 nm absorption peak. The single triangle indicates the value obtained by both CT and DIP-CT after 3 hours at pH 13°, 16°. The coordinates defining the solid curve were obtained from the line computed by the method of least squares in Fig. 3. This line applies to the reversible portion of the titration curve only.

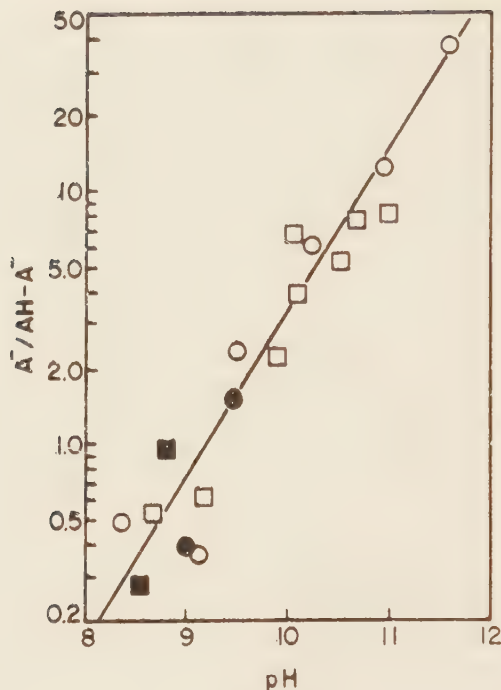


Figure 3

A plot of pH versus $\log A^-/(AH - A^-)$ for the data obtained in Figure 2. A^- corresponds to the ionized tyrosyl groups, and AH to the total concentrations of reversibly titrating tyrosyl groups. The open circles and squares are for the forward titration of CT and DIP-CT, respectively; the filled circles and squares are for the corresponding back titration.

authors previous experience with the enzyme, a brief account of the line of investigation, which has been followed by the author and his predecessors, will be given.

The first major milestone in the elucidation of chymotryptic catalysis was the finding by Schaffer and coworkers⁶⁹ that it was possible to isolate a stable chymotrypsin-substrate intermediate compound in the catalytic hydrolysis. These workers used the moderately specific substrate diisopropylfluorophosphate (DFP) and isolated the covalent acylenzyme, diisopropylphosphoryl-chymotrypsin (DIP-CT) in which the substrate fragment diisopropylphosphate was attached to a specific serine sidechain in the enzyme by an ester linkage. Subsequently, Hess and Wootton⁴⁰ characterized the structural difference between DIP-CT and CT and so discovered the existence of a difference spectrum near 290 nm between DIP-CT and CT (Fig. 1). The location of this difference spectrum on the wavelength scale indicated, that it arose from a perturbed aromatic sidechain. Chymotrypsin contains three types of aminoacids which could come in question as the origin of this spectral effect: Tryptophane, tyrosine and phenylalanine. Since the extinction coefficient of phenylalanine in this range is much smaller than that of

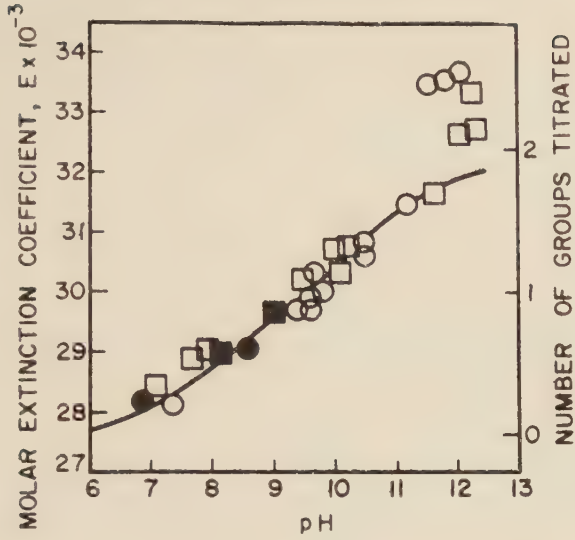


Figure 4

Spectrophotometric titration curve of CT and DIP-CT in 0.14 *M* KCl at 16° at a wavelength of 295 nm. The open circles and squares are for the forward titration of CT and DIP-CT, respectively, the filled circles and squares are for the corresponding back titration. The coordinates defining the solid curve were obtained from a line computed by the method of least squares.

This line applies to the reversible portion of the titration curve only.

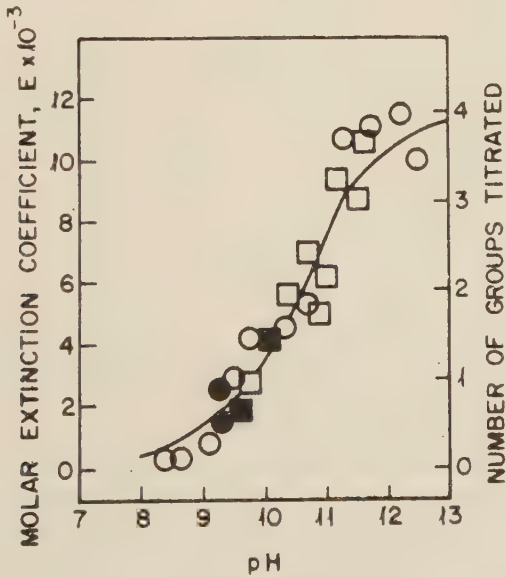


Figure 5

Spectrophotometric titration curve of CT and DIP-CT in GU at 16° C at a wavelength of 295 nm. The open circles and squares are for the forward titration of CT and DIPCT, respectively, the filled circles and squares are for the corresponding back titration. The coordinates defining the solid curve were obtained from a line computed by the method of least squares.

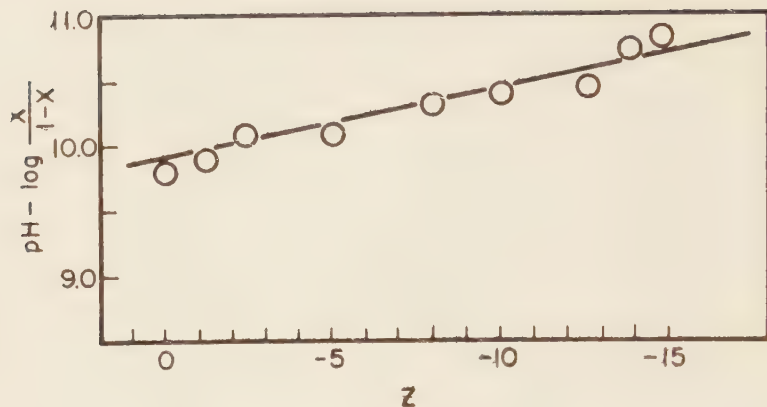


Figure 6

Plot of data for tyrosyl groups in GU. The values for $\log \left(\frac{X}{1-X} \right)$ were computed from the solid line of fig. 5. Z: Net charge of the protein. X: Fraction titrated.

tryptophane and tyrosine⁷⁰, the attention primarily was focussed on the latter two amino acids.

At first, the possibility of the involvement of tyrosine in the catalytic process was investigated. Hess and the author⁷¹ (see also ref. 168) found that two of the four phenol sidechains in α -chymotrypsin and diisopropylphosphoryl-chymotrypsin could be reversibly titrated in 0.70 and in 0.14 molar potassium chloride solution (Fig. 2-6). The apparent pK' -values of these four residues (two in CT and two in DIP-CT) were identical. After denaturation of CT and DIP-CT in a mixture of guanidinium chloride and urea, all the eight phenol groups present (4 in CT and 4 in DIP-CT) could be reversibly titrated and all displayed the normal pK' -value. Therefore, it was very unlikely that a tyrosine residue was the origin of the difference spectrum at 290 nm. Consequently, the work was concentrated on a characterization of the tryptophyl residues in CT and DIP-CT. By a simple model experiment, Oppenheimer, Mercouroff and Hess⁴² proved that the indole sidechain indeed could give rise to the observed difference spectrum.

Since no changes in the covalent structure of the enzyme accompanied the formation of DIP-CT from CT with the exception of the ester linkage of a specific serine sidechain, it appeared that the difference spectrum only could arise from a conformational change in the macromolecule. Much evidence from chemical modification of chymotrypsin and protolytic titration of CT and DIP-CT supported this contention¹. As the optical rotatory power of an asymmetric molecule was, and still is, widely accepted as a very sensitive indicator of conformational changes, it was decided to perform measurements of the optical rotatory dispersion of chymotrypsin and isolated acylenzymes. Unfortunately, the interpretation of such data is complicated by the possibility of errors from optical dichroism (anisotropic light absorption) and anisotropic light scattering. Even in the absence

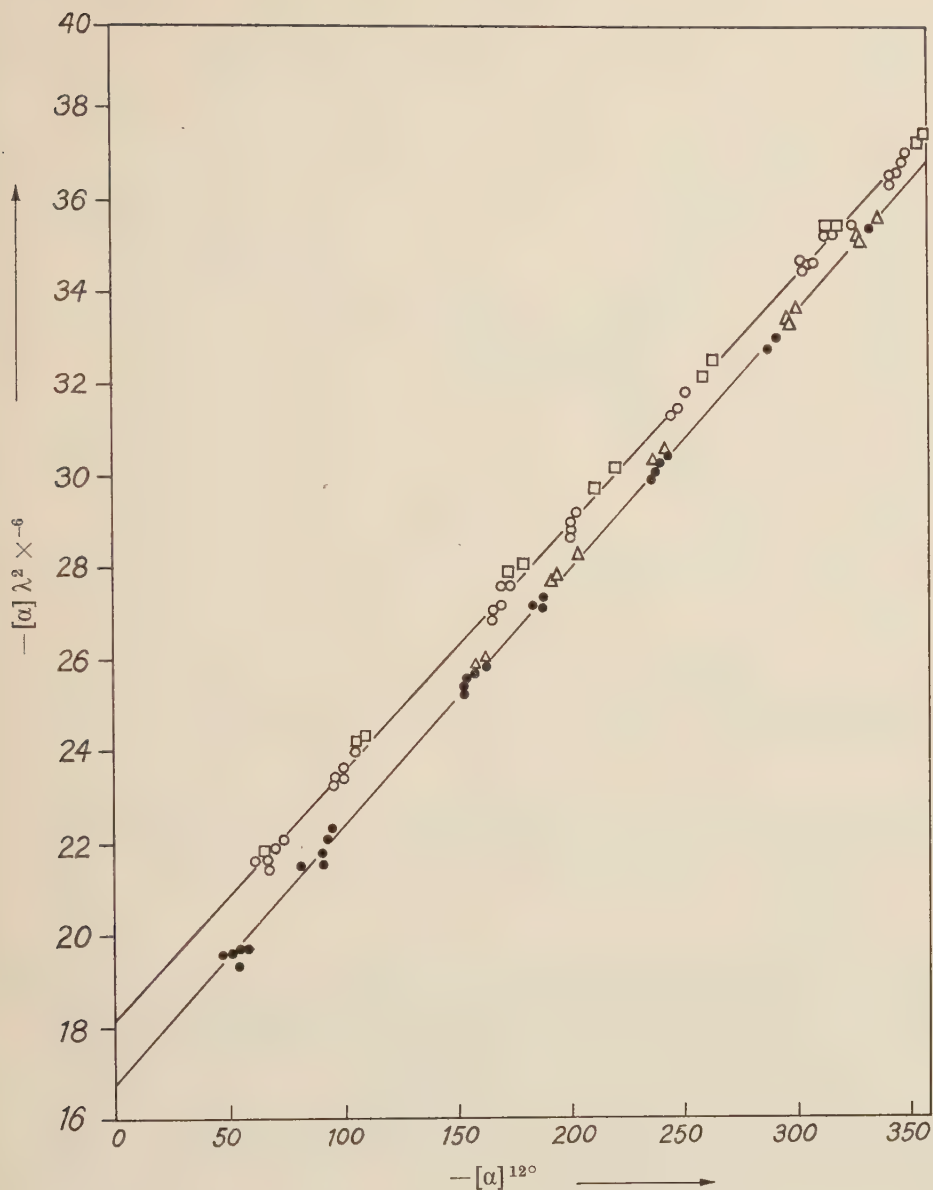


Figure 7

Modified Lowry plot of optical rotatory dispersion: α -chymotrypsin, $\circ$; diisopropyl-phosphoryl- α -chymotrypsin, $\bullet$; monoacetyl- α -chymotrypsin, $\blacktriangle$; and the latter after deacylation, $\square$; pH 3.8 (acetate buffer, $\mu = 0.14 M$), $12^\circ C$, 2 dm cell. Protein concentration: 0.08–0.15 g/100 ml. The solid lines were computed by the method of least squares using the data for α -chymotrypsin and DIP-CT, respectively.

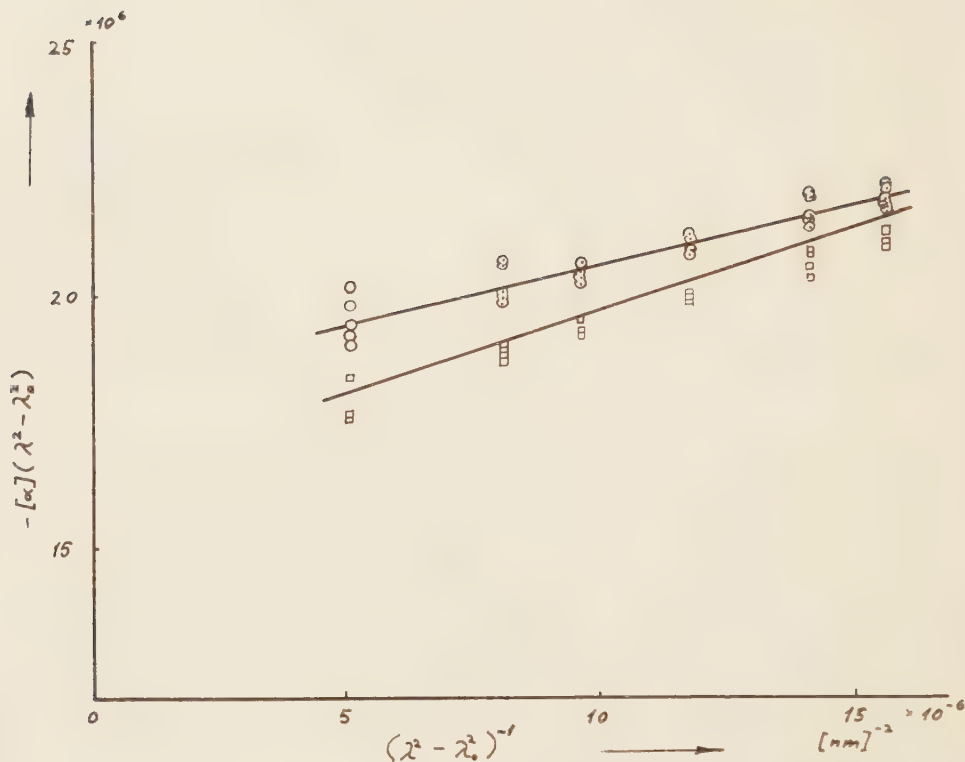


Figure 8

Moffitt plot of the data in Fig. 7. The lines were computed by linear regression. The circles represent α -CT and the squares DIP-CT.

of such errors, which are detectable⁷², ambiguity is possible, since the measured asymmetry characterizes the total environment of the perturbed electrons and therefore includes both the conformation of the macromolecule and the solvation of the center of asymmetry⁷³. The relative contribution of the various factors to the observed parameters is not fully understood¹⁶⁹, but considerable progress has been made and excellent results have been achieved by combination of optical, chemical and titrimetric evidence⁷⁴.

The optical rotatory dispersion parameters of α -chymotrypsin and deacylated monoacetyl-chymotrypsin proved to be identical⁸, but different from those of DIP-chymotrypsin and monoacetyl-chymotrypsin (Fig. 7 and 8). Hence, the acylation reaction was in both cases (DFP and p-nitrophenylacetate substrates) accompanied by a reversible change in dispersion parameters. The changes in the a_0 - and K' -parameters considered in conjunction with evidence from chemical and spectral studies and from the effect of a change in the properties of the solvent suggested, that the acylation process was accompanied by considerable changes in the solvation of the binding site and slight, if any, alterations in the

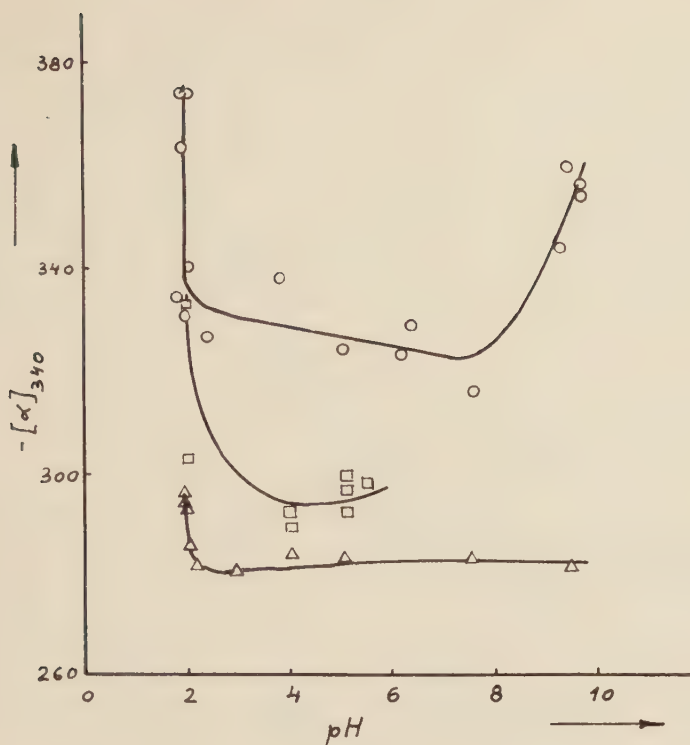


Figure 9

The pH-dependence of the specific rotation of α -CT, AcCT and DIP-CT of lot no. 6026 at 340 nm. The circles represent α -CT, the squares AcCT and the triangles DIP-CT. The curves are smooth lines drawn between the points to give the best fit compatible with the experimental error.

helical content. It was tentatively concluded, that the rearrangements in the solvent structure were results of conformational changes in the enzyme, and that these rearrangements led to great similarities in the three-dimensional structures of DIP-chymotrypsin and monoacetyl-chymotrypsin.

The specific rotation of α -chymotrypsin was, in contrast to that of DIP-chymotrypsin, strongly pH-dependent (Fig. 9). Monoacetyl-chymotrypsin occupied in this respect an intermediate position. The curves suggested the involvement of amino- and carboxyl groups in the catalysis and stability of the active site. This later has been strongly confirmed by the work of Labouesse, Oppenheimer and Hess who proved that the α -amino group of the N-terminal isoleucyl residue (B-chain) controlled the activity of the enzyme⁴⁶.

The elucidation of chymotryptic catalysis had then so far progressed, that a hypothesis, which seemed to be plausible, could be proposed: The binding of a specific substrate to chymotrypsin changed the pK' -value of the N-terminal isoleucyl residue and this elicited a conformational change (in the B-chain) which altered the environment of a specific tryptophyl sidechain. The rearrangement

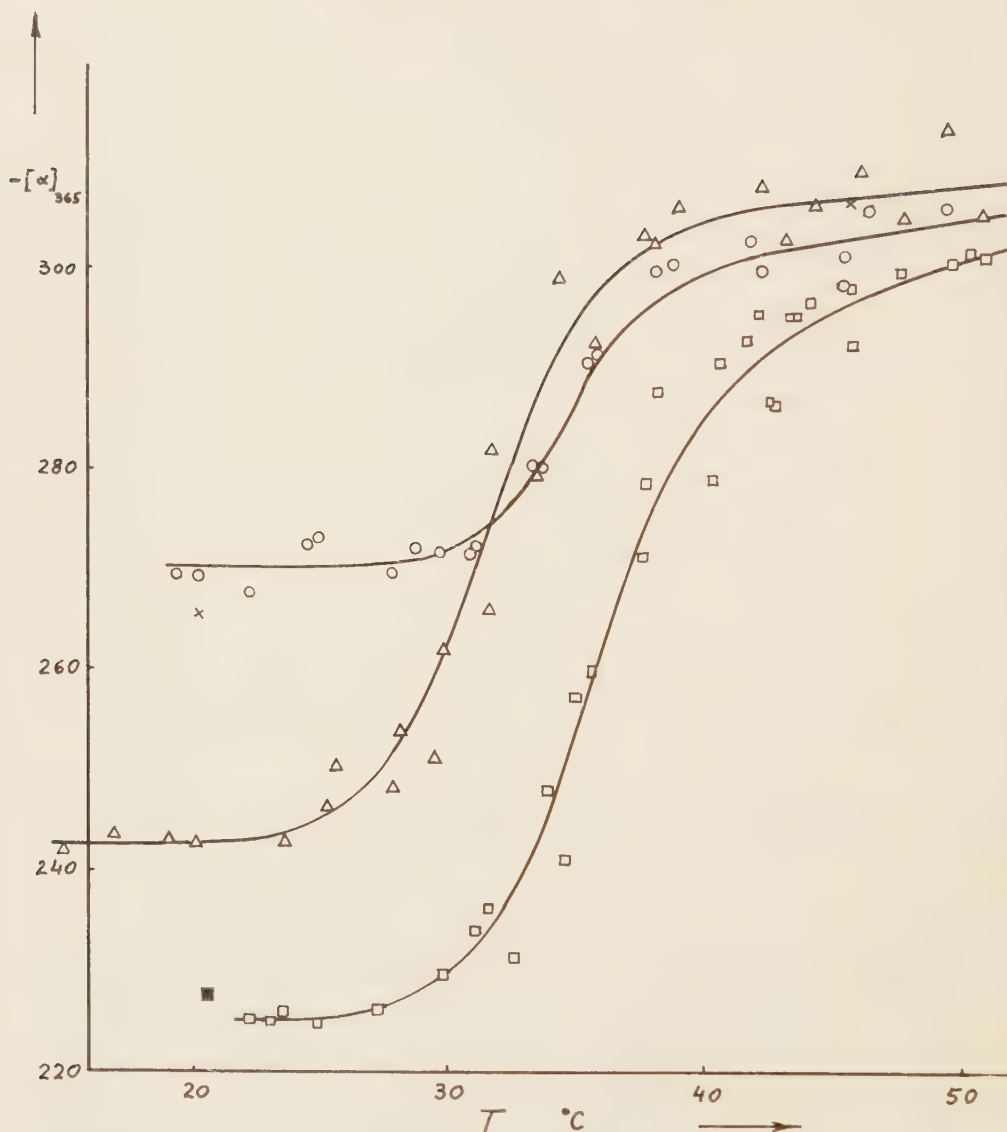


Figure 10

The temperature dependence of the specific rotation of α -CT, AcCT and DIP-CT at 365 nm. Symbols: $\circ$, α -CT, 3 cryst.; Δ , AcCT, corrected for free α -CT; $\square$, DIP-CT, from 3 cryst. α -CT; $\times$, α -CT, chromatographically pure; $\blacksquare$, DIP-CT, from chromatographically pure α -CT. Lot no. 6027. Readings within 30 min. of adjustment to a new temperature; pH 2.00; $\mu = 0.01 M$.

could be detected by the appearance of the difference spectrum which paralleled the acylation reaction.

Now, Hess and the author undertook a study of the stability of this "acyl fold". The thermally induced, reversible transitions of α -chymotrypsin, DIP-

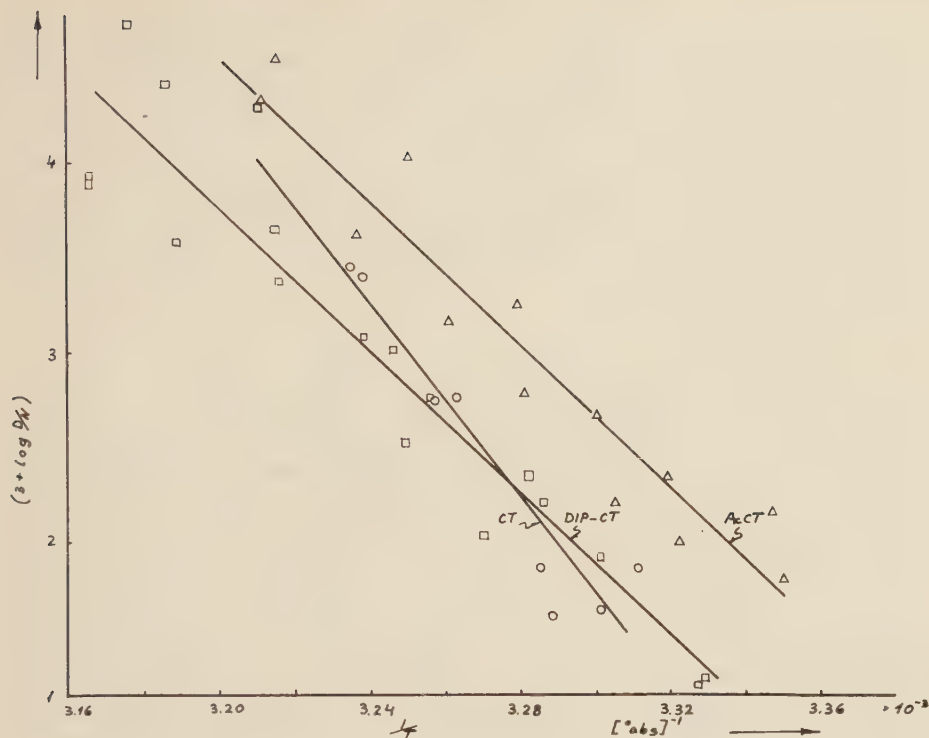


Figure 11

Van't Hoff plot for the data in fig. 10. Symbols: $\circ$, α -CT; $\square$, DIP-CT; $\triangle$, AcCT corrected for free α -CT; D: Molar fraction of the protein in the denatured (unfolded) conformation; N: Molar fraction of the protein in the native (folded) conformation.

chymotrypsin and monoacetyl-chymotrypsin were characterized at pH 2 and low ionic strength (necessary for reversibility) in the hope, that an evaluation of the thermodynamic parameters would throw some light upon the question of the mechanistic significance of the conformational change^{4, 164-167}. Besides, the effects of solvent polarity and ionic strength on the enthalpy of unfolding were determined. This information is useful, as it provides an indication of the bond types involved.

The conclusion was that α -chymotrypsin, DIP-chymotrypsin and monoacetyl-chymotrypsin undergo reversible, thermal transitions at 30–40° C, pH 2 and low ionic strength (0.01 *M*) (Fig. 10). The entropies and enthalpies of transition greatly vary from enzyme to acylenzyme and between the various acyl-enzymes (Fig. 11). Also the transition temperature significantly varies, but the changes are small. At temperatures above the transition range, all the three proteins almost have the same specific rotation. This indicates, that the acyl fold is opened in the course of the transition process and that the three proteins almost assume the identical structure at temperatures corresponding to completion of this unfolding reaction.

The presence of a low concentration (0.60 vol. percent) of propanol-2 in the solution markedly lowered the specific rotation of DIP-chymotrypsin at high temperature and raised the standard enthalpy of transition by about 40 kcal/mole to the level of that of the transition of α -chymotrypsin. Therefore, it was assumed that a structure characteristic of the acylation reaction by DFP had been eliminated by addition of the alcohol. The effect of changing the polarity of the solvent, the direction of the change in standard enthalpy of transition and the insignificant change in net charge indicated, that this structure to a large extent was stabilized by hydrophobic interactions.

The research plan of this thesis

The experimental and theoretical basis of the dissertation has been outlined above. The objective of the project was twofold: To ascertain whether or not the phenomenon of conformational changes in enzymes accompanying their catalytic action is general and to characterize the isomerizations in chymotrypsin while the enzyme is exercising its catalytic function. At the outset, it was expected that the structural rearrangements of chymotrypsin controlled the catalytic activity kinetically. However, certainty in this question requires the establishment of the entire kinetic scheme and measurement of the rate parameters of the elementary steps. Besides, alternate or supplementary functions of the conformational changes, such as steric control of the specificity and regulation of the equilibrium between active and inactive isomers of the enzyme, were possibilities which had to be explored.

Chapter 3

Experimental section

Characterization of conformational changes in
some catalytic proteins

3.1. CHYMOTRYPSIN

3.1.1. Changes in proton equilibria accompanying the isomerization of the enzyme

This work was carried out to characterize the conformational changes in greater detail and to attempt to localize them in the structure. Since the protein contains about thirty ionizing groups, the probability was high, that one or more were directly or indirectly involved in the rearrangement and hence influenced by this process. This expectation proved to be correct.

Preliminary experiments by Moon, Sturtevant and Hess²⁶, who measured the number of protons released on reaction of chymotrypsin with diisopropyl-fluorophosphate (DFP) at various pH-values using a protolytic indicator, disclosed a difference in the titration curves of about one proton per protein molecule. Its pK' -value was near 9. The present study has confirmed this finding and provided more details.

Electrometric titrations have previously been extensively used and with considerable success for elucidation of specific structures in proteins. Notable workers in this field are Linderstrøm-Lang⁷⁵, Wyman⁷⁶, Edsall⁷⁶, Kirkwood⁷⁷, Tanford^{77, 78} and Scheraga⁷⁹.

Recently, Marini and Wunch⁸⁰ reported similar titrations. They investigated α -chymotrypsin and chymotrypsinogen in water, 20% aqueous formaldehyde and in 8 molar urea, and also attempted by differential methods to detect the changes in proton equilibria on chymotrypsin following acylation of the active site. These measurements were complicated by extensive autolysis of the enzyme. Corrections for this were made using the measured rate of autolysis. Such a procedure, unfortunately, is very difficult to use, since the corrections are larger than the effect. It is preferable to avoid appreciable autolysis by rapid titration or by use of a "point by point" method. Both of these methods were used in the present work. Although the effect is rather small, it was invariably found in a large number of experiments (about 25) and independently confirmed in a different laboratory.

Experimental Procedures

Electrometric Titrations

Electrometric titrations of α -chymotrypsin and diisopropyl-phosphoryl-chymotrypsin were carried out in 0.15 molar and in 0.40 molar potassium chloride and in eight molar urea at 4, 16 and 25° C using a Radiometer automatic titration assembly (Autotitrator TTT1 and Titrigraph

SBR2) with a calibrated All-glass Agla-syringe from Burroughs-Wellcome, England. Certain titrations were carried out manually using the same type of syringe in a micrometer outfit from Wellcome Research Laboratories, Beckenham, Kent, England. The electrodes were a general purpose (B) glass electrode and a fiber type calomel electrode, both from Radiometer, Copenhagen. The enzyme solutions were titrated in a glass vessel with a jacket through which water of constant temperature circulated. A thermostat bath from Haake Gebrüder, Berlin (model F) controlled the temperature. Excess heat was removed from the thermostat bath by a heat exchange spiral cooled with a flow of a water-ethylene glycol mixture from a refrigerated reservoir (0–15° C). Vessel and tubes were insulated to permit a temperature control of better than $\pm 0.05^\circ$ C. The top of the titration vessel was covered by a loosely fitting plexiglass cover with holes for the electrodes and inlet for nitrogen. A stream of nitrogen, saturated with water to reduce loss by evaporation, was constantly led over the surface of the enzyme solution to expel carbon dioxide from the vessel.

The syringes were calibrated by weighing with water of known temperature. The proportionality between the distance travelled by the plunger and the volume of water extruded was very good. Calibration of the recorder was carried out by titration of a known volume of standard acid with standard base. The linearity between the volume of base added and the indicated voltage change on the recorder was very good. This ensures that the method of registration is adequate.

Ultraviolet Absorbance

The spectral measurements were made with a Cary 14 continuous spectrophotometer from Applied Physics Corporation, Monrovia, California.

Chemicals

Alpha-chymotrypsin was a three times crystallized, salt-free (CDI-grade) product from Worthington Biochemical Corporation, Freehold, New Jersey, USA (lots no. 6027 and 6031). The enzyme was tested for activity and salt content and used without further purification since our laboratory has had many years of good experience with the product. The protein concentrations were measured spectrophotometrically at 280 nm using a molar extinction coefficient⁸¹ of $50000 \text{ cm}^{-1} \times 1 \times \text{M}^{-1}$. The molecular weight was assumed to be 25000^{82} .

Diisopropyl-phosphoryl-chymotrypsin (DIP-CT) was prepared as described by Jansen, Nutting, Jang and Balls⁸¹. The samples (110 mg in 10 ml water) were exhaustively dialyzed 24 hours at 4° C against 4 changes of 6 liters of 7×10^{-4} molar hydrochloric acid. Phosphorus analysis by the method of Summer⁸³ and the difference spectrum: DIP-CT versus α -chymotrypsin (α -CT) were used to check the preparation⁴⁰.

All other chemicals were analytical grade (Mallinckrodt) unless otherwise indicated. The diisopropyl-fluorophosphate was purchased from K&K Biochemicals Co. The urea was of analytical grade (Mallinckrodt) and had the correct melting point (was checked).

Carbon dioxide free base (potassium hydroxide) was prepared by the method of Kolthoff⁸⁴. The purified base was standardized against potassium phthalate (ACS reagent grade from B&A Co.). The hydrochloric acid solution was diluted and standardized against the base.

The solutions used to calibrate the pH-meter were Beckmann pH 7 buffers and 0.01 molar borax solution prepared as described by Bates⁸⁵. The temperature coefficients of the pH-values of the buffers were also taken from the work of Bates. The pH-meter (TTT1) was checked with the buffers before and after each experiment and a rapport better than ± 0.015 pH-unit was found. The observed pH-values were corrected for scale error using linear corrections.

All solutions were prepared with distilled, deionized and freshly outboiled water.

The Titrations

The freshly prepared enzyme solutions were dialyzed as described above and diluted to 100 ml in a volumetric flask to give a concentration of 1 mg enzyme

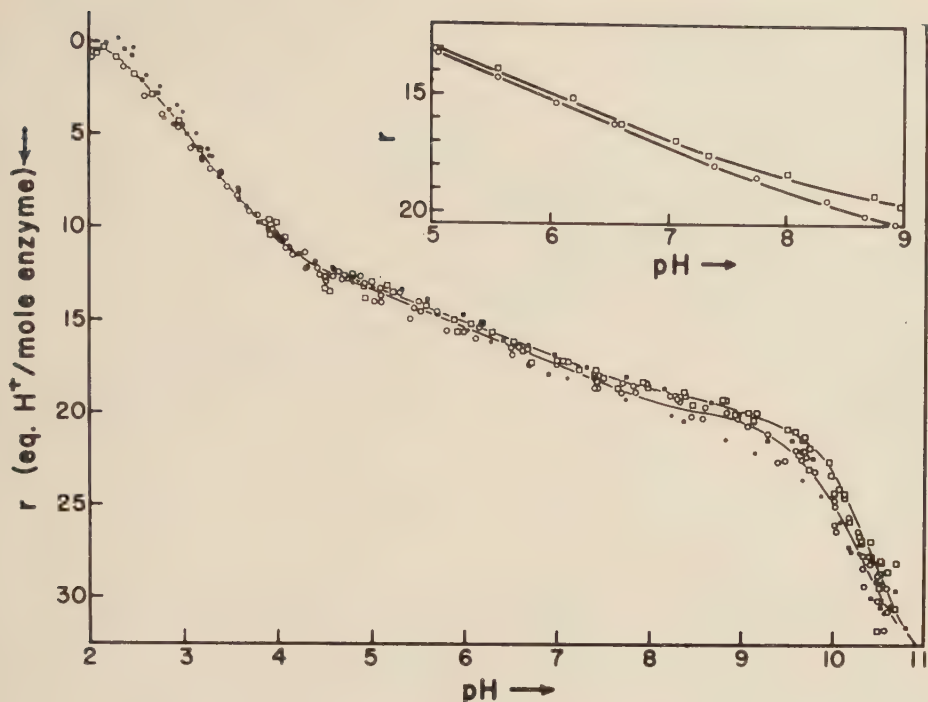


Figure 12

Electrometric titration of α -CT and DIP-CT at 25° C in 0.15 molar KCl solution. Enzyme concentration: 1 mg/ml. α -CT: $\circ$; DIP-CT: $\square$. Forward titration: open symbols; reverse titrations: filled symbols. The curve is theoretical (see text) in the pH-range: 1.5–5 and the best fitting smooth lines which could be drawn between the points at pH-values above 5.

Inset: Enlargement of the graph in the range of greater interest. Each point in the inset represents the density center of a cluster of observed values.

per ml in the required solvent. Ninety milliliters were transferred with a pipette to the titration vessel and allowed to reach thermal equilibrium (15–30 min.) The pH in the salt solutions of the enzyme was invariably 4.00 ± 0.06 . Automatic forward titration was made as soon as possible to pH 10.5 to minimize any denaturation or autolysis. The titration was intermittently interrupted to check the correspondence between the recorder and the pH-meter. When pH 10.5 was reached, back titration to pH 3.5 was performed within one minute using manual titration with the micrometer syringe and standard acid. A new automatic forward titration was then made to pH 11.5 followed by manual back titration to pH 4.

The extreme pH-ranges: 1.5–4 and 10.5–11.5 were also studied using a small initial volume (10 ml) and high protein concentrations (4 and 13 mg/ml) to reduce the solvent error. The low enzyme concentration in the pH-range of most interest (4–10.5) was chosen to reduce possible influence from the aggregation reaction which was expected. The large quantities of enzyme reduce the titration error.

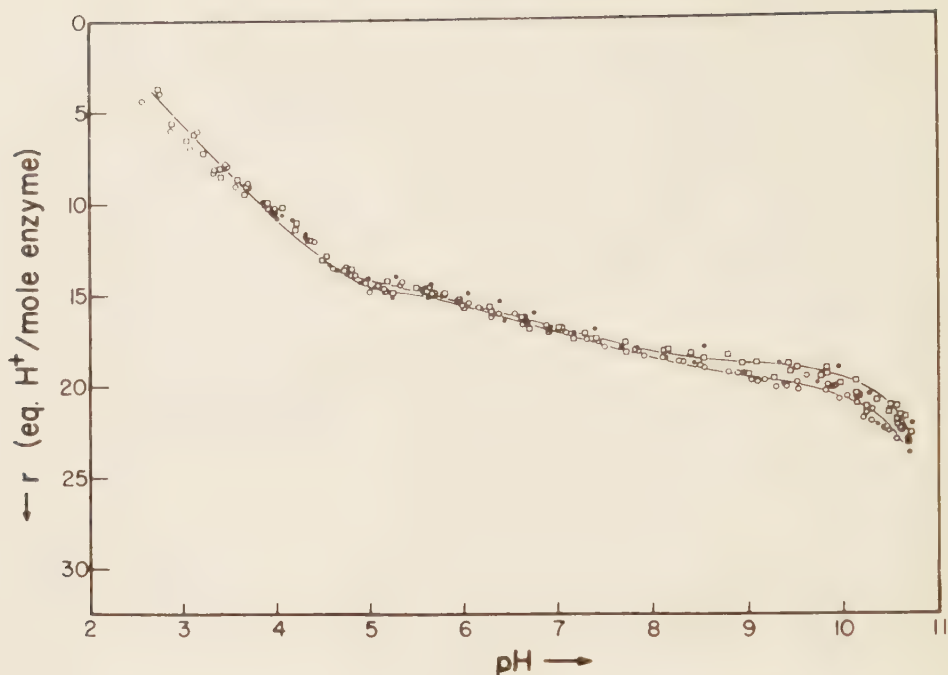


Figure 13

Electrometric titration of α -CT and DIP-CT at 4° C in 0.15 molar KCl solution. Enzyme and symbols as in Fig. 12.

Activity of the Enzymes

The activity of α -CT and DIP-CT toward N-acetyl-L-tyrosine ethyl ester (ATEE) was measured as previously described⁸. The following apparent first order constants were found:

α -CT	Lot no. 6027: $k = 106.0 \text{ sec}^{-1}$
	Lot no. 6031: $k = 103.5 \text{ sec}^{-1}$
	Chromatographically pure: $k = 120 \text{ sec}^{-1}$
DIP-CT	Lot no. 6027: $k = 0.66 \text{ sec}^{-1}$
	Lot no. 6031: $k = 0.53 \text{ sec}^{-1}$, i.e. less than 0.5% of the native activity of α -CT remained.

These activities are common for such preparations and were considered to be satisfactory.

Results

Titration curves were obtained for α -CT and DIP-CT in the pH-range from 1.5 to 11.5 in 0.15 molar potassium chloride solution at 4, 16, and 25° C and in 0.40

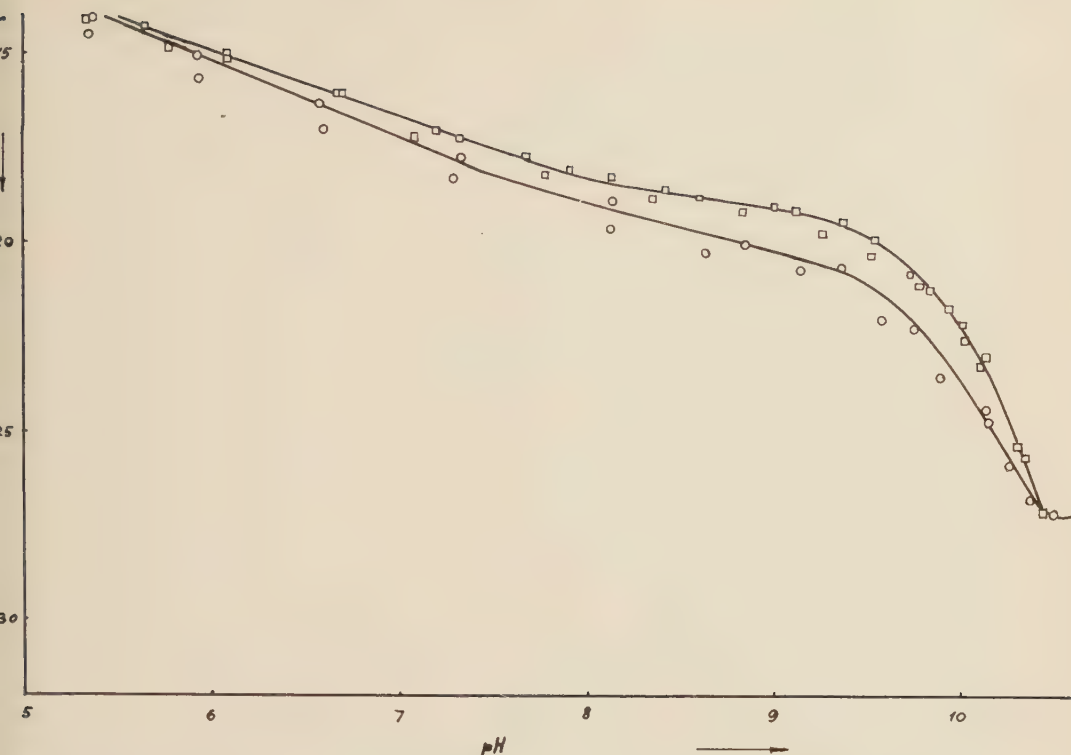


Figure 14

Electrometric titration of α -CT and DIP-CT at 16° C in 0.15 molar KCl solution. Enzyme concentration and symbols as in Fig. 12.

molar KCl solution at 4 and 16° C. (Fig. 12–14 and 16). Besides, titration curves for the two enzymes were determined in 8 molar urea (Fig. 15) at 25° C (lower pH-limit: 2.5). According to common usage in the field of protein chemistry, the number of protons per enzyme molecule titrated since the state of maximal protonation, r , was plotted as a function of pH⁷⁶. At 4° C, the initial pH of the solution of CT and DIP-CT was the same as that at 25° C. It was therefore assumed that the isoionic point remained unchanged when the temperature was lowered from 25 to 4° C. Consequently, the function r was calculated from r' , which is the number of protons titrated per molecule of enzyme since the dissolution, by addition of 10.5. The latter figure represents the number of protons per enzyme molecule titrated from the state of maximal protonation to the pH-value which is observed on dissolution of the enzyme in 0.15 molar KCl.

The titration curves were reversible in the pH-range: 3.5–10.5 under all the conditions used. Above pH 10.5, irreversible changes occurred which released more protons per protein molecule than the number corresponding to the conditions of reversability. A concomitant increase in absorbance in the ultraviolet

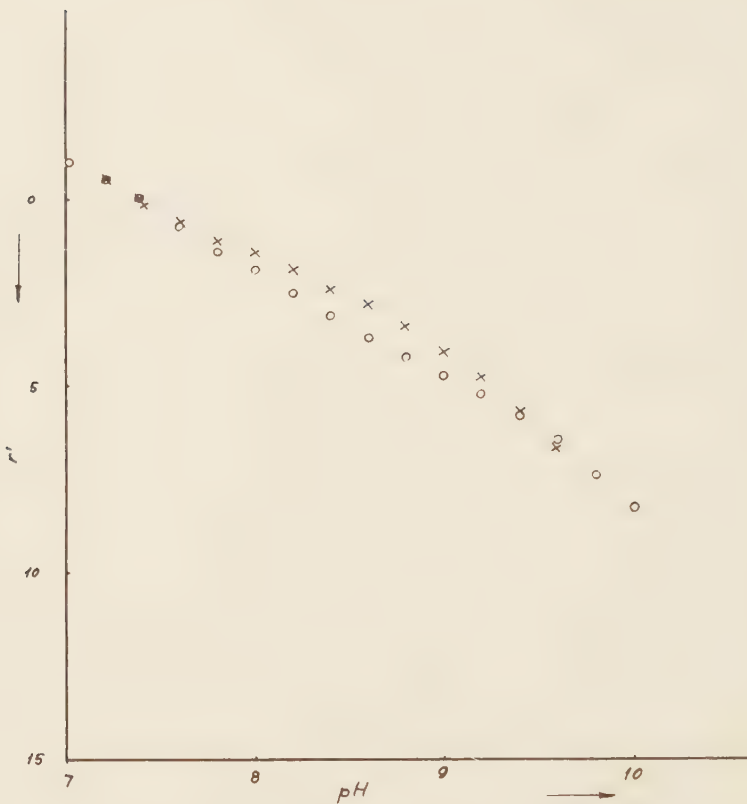


Figure 15

Electrometric titration of α -CT and DIP-CT at 25° C in 8 molar urea, 0.15 molar KCl solution. α -CT: o; DIP-CT: x. Enzyme concentration: 1–1.3 mg/ml. r' = the number of protons titrated per protein molecule since pH 7.38.

range, loss of activity and a change in the apparent dissociation constant for two phenol groups (tyr) were observed. Exposure of the enzymes to acidities below pH 2 also caused irreversible changes in structures influencing proton dissociation constants resulting in the production of additional 1–2 protons per enzyme molecule at pH 4–10. The same observation was made during titration of α -CT after the enzyme had been allowed to autolyze.

It may be seen on fig. 12 and 13 that the general course of the titration curves of α -CT and DIP-CT is similar, but that about one proton per enzyme molecule is titrated in the former, but not in the latter protein.

The isoelectric point reported by Alberty and coworkers⁸⁶, 8.6, was (in the absence of other data) used as isoionic point for both enzymes. This is not strictly correct, but since the two molecules are very similar, because the extra group does not carry a charge or eliminate one and since chloride binding in all other known cases of protein-chloride interaction has been negligible in this pH-range, the

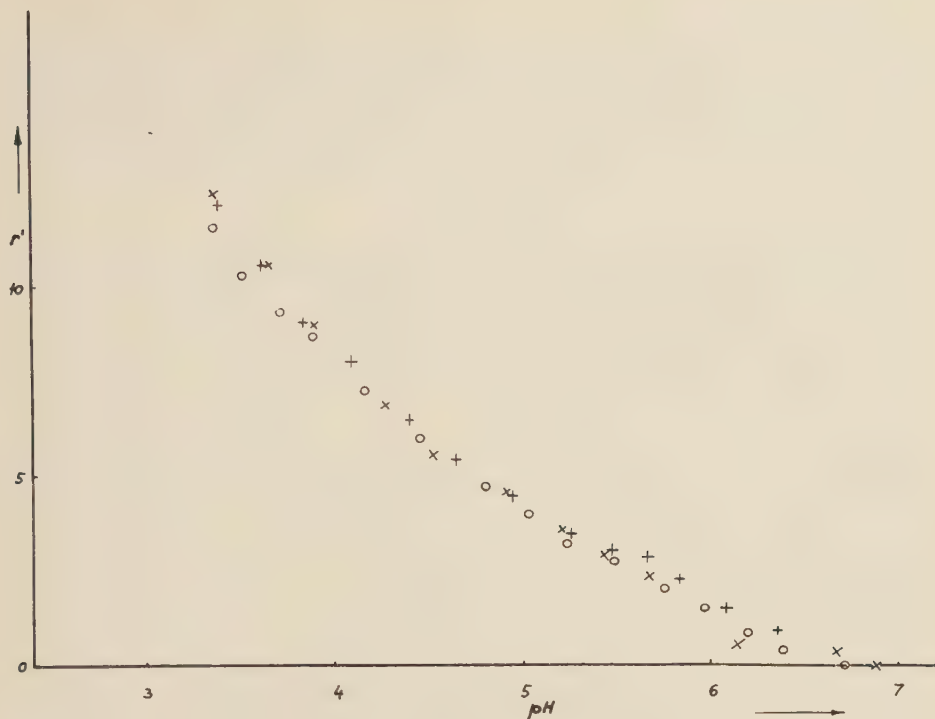


Figure 16

Electrometric titration of α -CT at 4°, 16° and 25° C in 0.40 molar KCl solution. Symbols: at 25° C: +; at 16° C: ○; at 4° C: ×.

Enzyme concentration: 0.94–1.05 mg/ml. r' = the number of protons titrated per enzyme molecule since pH 7.00 in 0.40 molar KCl at the indicated temperature.

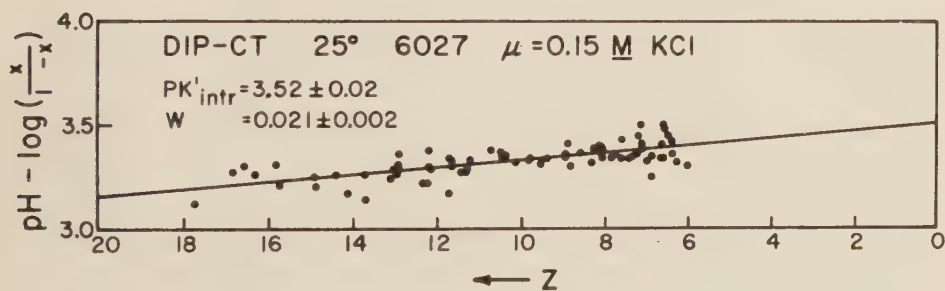


Figure 17

Graph for correction for general electrostatic effect on titration data of DIP-CT (from CTlot. # 6027) in 0.15 M KCl solution at 25° C. The values for $\log(X/(1 - X))$ were computed from the data in Fig. 12. Z: net charge of the protein. X: molar fraction titrated. W = electrostatic factor.

assumption was considered to be permissible⁸⁷. Deionization on an ion exchange column such as Dintzis' has been used⁷⁸, but it involves danger of creation of artifacts due to acid or base catalyzed rearrangements.

The correction for general electrostatic effect on the ionization of the carboxyl

groups (13 groups per molecule) in both proteins was made as previously described⁷¹. The results are presented in Table 1 and on Fig. 17. The theoretical curves based on the experimental data in Table 2 excellently fit the observed values in the pH-range: 2-5 (shown on Fig. 12 and 13). Outside this range, the

Table 1

Parameters characterizing the ionization of protolytic groups in α -CT and DIP-CT in the carboxyl range (25°C, 0.15 M KCl).

Enzyme	w	pK'_{intr}	n
α -CT.....	0.012 ± 0.002	3.44 ± 0.05	13
DIP-CT.....	0.021 ± 0.002	3.52 ± 0.05	13

n = the number of carboxyl groups required to make the theoretical curve fit the experimental points, (see Fig. 17).

pK'_{intr} = the logarithm (with negative sign) of the intrinsic ionization constant of carboxyl groups in the protein.

w = the electrostatic factor.

Table 2

Titration of α -CT in 0.40 molar KCl solution at 4°, 16° and 25°C.

4°C		16°C		25°C	
pH	r'	pH	r'	pH	r'
6.88	0.00	6.71	0.00	6.69	0.00
6.67	0.42	6.39	0.43	6.36	0.96
6.14	0.56	6.20	0.88	6.09	1.54
5.67	2.37	5.97	1.53	5.84	2.32
5.43	2.98	5.76	2.04	5.67	2.90
5.21	3.66	5.49	2.78	5.48	3.09
4.91	4.66	5.24	3.27	5.26	3.53
4.53	5.60	5.03	4.01	4.95	4.58
4.27	6.95	4.80	4.78	4.65	5.49
3.90	9.07	4.45	6.04	4.40	6.57
3.66	10.67	4.16	7.31	4.09	8.07
3.37	12.51	3.89	8.78	3.84	9.15
		3.71	9.37	3.62	10.61
		3.52	10.34	3.39	12.21
		3.37	11.60		

r' = the number of protons titrated per enzyme molecule since dissolution in 0.40 molar KCl solution at the indicated temperature.

Initial enzyme concentrations: at 4°C: 0.997 mg ml.

at 16°C: 0.943 mg ml.

at 25°C: 1.048 mg ml.

The molecular weight is assumed to be: 25000.

curves on the graphs represent averages of three independent experiments. The negative slope in the plot for correction of general electrostatic effect outside the range of the carboxyl groups and the consequent failure of the titration theory indicate the occurrence of conformational changes in the enzymes during the titrations. The pH-dependence of the specific rotation, mentioned above, supports this view.

The rates of autolysis were measured and kept insignificant by confining the experiments to relatively short periods of time which still permitted attainment of chemical and thermal equilibrium. The enzymatic activity and the phosphorus content were checked after each experiment and the loss in the reversible range was found to be negligible. The exclusion of carbon dioxide was tested and found to be effective.

The standard error in the titration curves was estimated to ± 0.15 proton per protein molecule and ± 0.002 pH-unit.

Discussion

The titration curve for α -CT in general agrees with that found by Marini et al.⁸⁰. The difference between the titration curves of α -CT and DIP-CT is highly significant (see Fig. 12 and 13) and is confirmed by work in a different laboratory. The pH-range in which the difference occurs (8–11) and its maximal value of about one proton per molecule of protein at pH about 9.5 suggest that amino or phenol groups are involved. Guanidyl and histidyl residues are unlikely participants in the phenomenon since they have not been found to ionize in this range in proteins. Involvement of tyrosyl groups is also unlikely since these residues appear to be identical in α -CT and DIP-CT judging from spectrophotometric titration curves⁷¹. The difference in titration curves persists under all conditions tested, including eight molar urea. Recently, Labouesse and coworkers published work on the properties of fully acetylated chymotrypsinogen and on the enzyme which is formed on its activation⁷⁴. They concluded that the difference titration curve, the difference spectrum in the 290 nm range, the catalytic activity of the enzyme and its conformation depended on the state of ionization of the N-terminal isoleucyl amino group which appeared on activation of the precursor. Electrostatic forces changed the conformation according to the charge on this group. The rearrangement in turn (reversibly) altered the polarity of the environment of a tryptophyl residue. All known data appear to be compatible with and strongly supporting this conclusion.

The graph in Fig. 17 shows that all the 13 carboxyl groups in α -CT or its DIP-derivative ionize with the same pK'_{intr} -value (3.5). This figure, which is anomalously low, may perhaps be explained by local electrostatic effects (the molecule is known to be a dipole), hydrophobic zones (low dielectric constant) or intramolecular hydrogen bonding⁸⁸. The observed difference in the electrostatic factors, w , between α -CT and DIP-CT is significant and worth noting. It seem to

indicate that a change in the location of charges has occurred during the acylation process.

The failure of the Linderstrøm-Lang equation and thus calculation of theoretical titration curves above pH 5 may be due to protein polymerization involving groups in this range. Isomerizations induced by the pH-changes could also be important.

Conclusions

1. Electrometric titration curves were obtained for α -chymotrypsin and diisopropyl-phosphoryl- α -chymotrypsin in the pH-range 1.5–11.5 in 0.15 molar KCl solution at 4°, 16° and 25° C, in 0.40, molar KCl solution at 4° and 16° C and in 8 molar urea, 0.15 molar KCl at 25° C. Reversability was found at pH 3.5–10.5. In all cases, the same difference between the titration curves of the two enzymes appeared in the pH-range: About 8 to about 10.5, with the greater difference occurring at about pH 9.5. The structure exhibiting this effect is not eliminated after two hours in 8 molar urea at pH about 6 and room temperature.

2. In both enzymes (CT and DIP-CT), all thirteen carboxyl groups ionized with the same pK'_{intr} -value which was anomalously low (3.5, while it normally is 4.0–4.5 in proteins). Whereas the pK'_{intr} -values of α -CT and DIP-CT were identical the electrostatic factor, w , significantly differed.

3.1.2. The specificity of the rearrangement

The purpose of this study was to determine the specificity of the interaction and to attempt to detect conformational changes in the enzyme accompanying the binding process by a kinetic method. Variation of the structure of the competitive inhibitor and a correlation of the binding and rate parameters with the steric and electronic characteristics of the inhibitor should provide a “negative print” of the binding site in 3 dimensions. Simultaneously, the data should give information about the specificity of release of the conformational changes reported by Hess⁷⁴ and others.

Since the conformational changes in the enzyme were to be coupled to the ionization of the α -amino-group of the N-terminal isoleucine residue, which has a pK' of about 9 in the free enzyme, the pH-dependence of the binding kinetics was determined. If this amino group through its charge acts as a “catch” in the molecule, then this reversible control mechanism should reach its maximal efficiency when pH is equal to this pK' -value. The pK' of the amino-group could be displaced by binding of a specific substrate, either directly by a change in the polarity of the environment or indirectly, through hydrophobic interactions which release a conformational change. The latter in turn changes the dielectrical constant near the N-terminus.

The following substances were chosen for the binding studies because of their structural similarity to substrates of chymotrypsin:

1. β -phenyl-propionate (hydrocinnamate).
2. N-acetyl-3,5-dinitro-L-tyrosine.
3. Proflavine (3,6-diamino-acridine).
4. Acriflavine (10-methyl-3,6-diamino-acridine).
5. Acridine.
6. 9-Amino-acridine.
7. Acridine orange (3,6-di (dimethyl-amino)-acridine).

The structural formulae are shown in Fig. 18. The relevant pK' -values are indicated. The substances listed (except 6) are known to possess binding specificity for chymotrypsin (stoichiometric binding, 1:1) and to be competitive inhibitors⁸⁹. The remaining compound is so closely related to the known inhibitors that it also was likely to be a competitive inhibitor. Contrary to expectations, it was found that 9-amino-acridine enhanced chymotryptic catalysis of the hydrolysis of hippurate methyl esters and related substrates. The activation increased with increasing size of the acyl-sidechain⁹⁰.

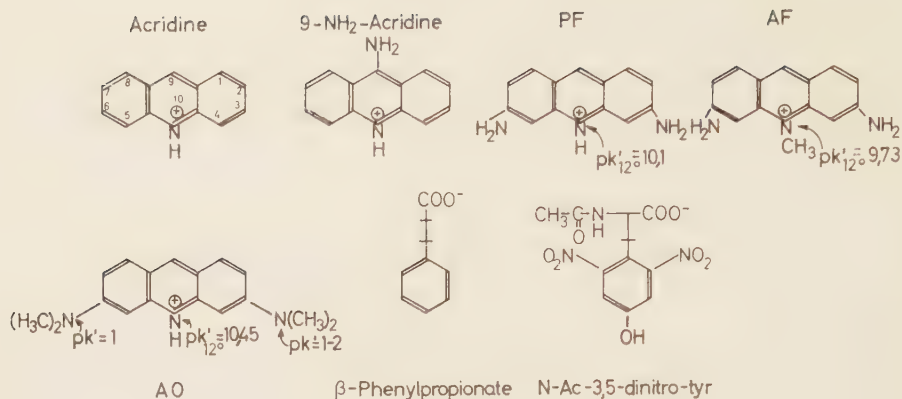


Figure 18
Structural formulae of the substrate models used.

It is yet unknown, which groups on the enzyme are determining the specificity of the interaction with substrates. Therefore, an attempt was made to find a model system of the enzyme which displayed the same selectivity for substrates as the biological catalyst. Indole was chosen as a simple model which might possess binding specificity. The reason for this choice was the observation of Hess and others^{40, 42}, that tryptophane is part of the binding site of chymotrypsin, trypsin and chymopapain.

The most useful of the inhibitors investigated (Fig. 18) was proflavine because of the suitable binding constant (about 10^5 l/moles) and the large spectral shift in a convenient range (about 450 nm) which occurs when the enzyme-inhibitor complex is formed. Therefore, the properties of proflavine has been extensively investigated in connection with enzyme reactions. Wallace et al.⁹⁰ have shown that proflavine is a highly effective inhibitor of chymotryptic catalysis of the hydrolysis of N-acetyl-L-valine methyl ester. This agrees with the observation of Bernhard and Lee⁹¹ that the binding could be completely prevented by addition of trans-cinnamoyl-imidazole. These authors also showed that the spectral shifts produced on complex formation with the enzyme can be mimicked by transferring proflavine from an aqueous solution to highly nonpolar solvents. The chromophore of this dye is probably perturbed in a similar way by non-polar parts of the protein.

The competition between proflavine and specific amide and ester substrates for the active site of chymotrypsin was used by Brandt and Hess⁹² to determine the overall binding constants of these substrates. Masking of the catalytically essential histidine residue(s) in chymotrypsin by specific alkylation with phenyl-methane sulfonyl chloride or L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone prevented binding of proflavine⁹³. This probably indicates that the binding and catalytic sites of the enzyme are closely connected which, in light of much other evidence, is a plausible hypothesis.

Bernhard and Gutfreund⁶² found that also trypsin binds proflavine. The affinity of this reaction is comparable to that found for chymotrypsin. The dye inhibited the tryptic catalysis of the hydrolysis of benzoyl-L-arginine ethyl ester competitively. These findings point to the existence of a strong structural resemblance between the binding sites of chymotrypsin and trypsin. The precursors, chymotrypsinogen and trypsinogen, failed to bind the dye. Therefore, at least the binding site for substrate is uncovered by the activation reaction.

Experimental

Materials: The enzyme (CT) was a 3 times crystallized, saltfree product from Worthington Biochemical Corporation (CDI). The activity of the enzyme was checked by the method of Schwert et al.⁹⁴ with N-acetyl-L-tyrosine ethyl ester as substrate.

Proflavine was purchased as hemisulfate from British Drug House and twice recrystallized from aqueous methanol. The substance was then chromatographically homogeneous. Acriflavine was purchased from Mann Research Laboratories Inc. (NF) and purified as PF by 2 recrystal-

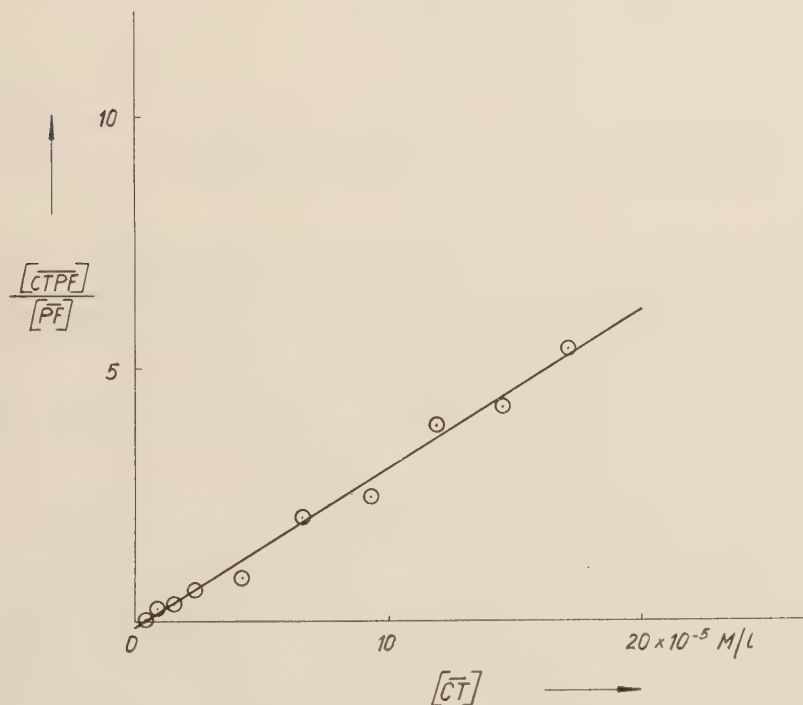


Figure 19

Result of spectrophotometric titration of proflavine with chymotrypsin at 11° C, pH 6.7 and 0.10 M ionic strength (phosphate buffer). The spectra were measured in the range: 340–600 nm. Absorbancies at 475 nm were used. The slope of the line = binding constant = $K = [\text{CTPF}]/[\text{PF}] \times [\text{CT}]$. The equation: $A^{475} = \epsilon_{\text{PF}}^{475} [\text{PF}] + \epsilon_{\text{CTPF}}^{475} [\text{CTPF}]$ was used with the experimental extinction coefficients: $\epsilon_{\text{PF}}^{475} = 5380 \text{ cm}^{-1} \times 1 \times \text{M}^{-1}$ and $\epsilon_{\text{CTPF}}^{475} = 16980 \text{ cm}^{-1} \times 1 \times \text{M}^{-1}$. Stated error: Standard deviations.

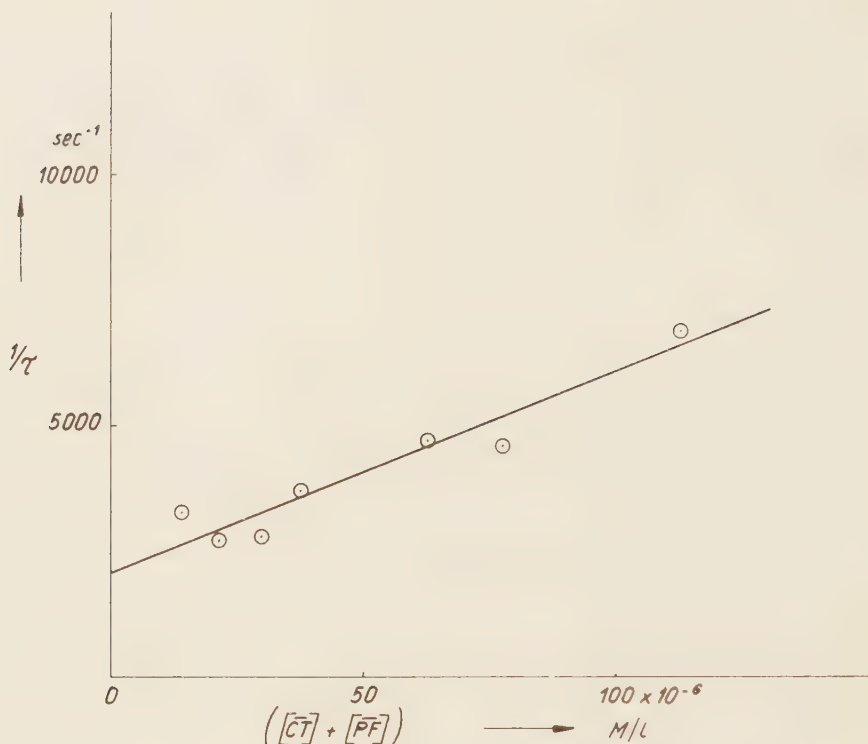


Figure 20

The concentration dependence of the relaxation rate ($1/\tau$) for the interaction of CT with PF at pH 6.7, 12° C and 0.10 M ionic strength (phosphate buffer). Slope: k_R . Intercept on the ordinate axis: k_D ; $[\text{CTPF}]/([\text{CT}] \times [\text{PF}]) = k_R/k_D$. Error stated: Standard deviations.

lizations from aqueous methanol. Acridine orange was a gift from Dr. F. Schneider of our laboratory. Acridine was purchased from Schuchart GmbH, München. The 9-amino-acridine was a product of Fluka (purum). All acridine dyes were purified as PF and tested chromatographically in aqueous methanol at alkaline pH for homogeneity.

The β -phenyl-propionate was purchased from Eastman Organic Chemicals. The N-acetyl-3,5-dinitro-L-tyrosine was a chromatographically pure product (CP) of Mann Research Laboratories. The buffers and salts were analytical reagents ("reinst") from Merck. All solutions were prepared with redistilled, degassed water.

Instrumental: The chemical relaxation experiments were carried out with the temperature-jump apparatus of Czerlinski and Eigen^{95, 96}. The instrument consists of a sample vessel with electrodes, an optical bench and a high-voltage discharge line. The reaction system was equilibrated in the thermostatted observation cell at 4° C for 10–15 min. The temperature was then brought to 12° C by a surge of current within a few microseconds. The resulting reequilibration at 12° C, the chemical relaxation, was followed spectrophotometrically at 204 nm (Schott interference filter with bandwidth 14 nm). The absorbancy changes were recorded on a Textronix oscilloscope and the curve displayed was photographed (Fig. 24). The relaxation time was evaluated from the slope ($0.434/\tau$) of the curve: $\log y$ versus time, where y is the measured photovoltage.

The pH was measured at 12° C with a Radiometer 22r pH-meter after calibration at 12° C with standard buffers (NBS)⁹⁶. The spectra were recorded with a Beckman DK2A ratio recording spectrophotometer using cuvettes with thermostat control.

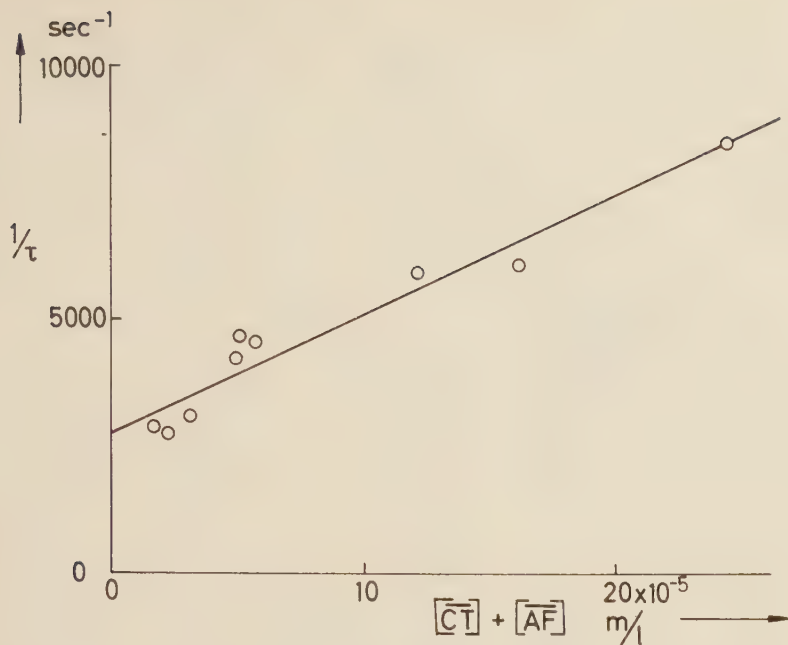


Figure 21

The concentration dependence of the relaxation rate for the interaction of CT with AF at pH 6.7, 12° C and 0.10 *M* ionic strength (phosphate buffer). Slope: k_R . Intercept on the ordinate axis: k_D . Error stated: Standard deviations.

The concentration of the inhibitor was $(1 - 2) \times 10^{-5}$ *M* and of the enzyme: $(0.5 - 25) \times 10^{-5}$ *M*. All the inhibitors, except β -phenyl-propionate, absorb visible light. One of the bands, which are altered in position and intensity on binding to the protein, is used to follow the reaction. In the case of β -phenyl-propionate, the pH-indicator chlorphenol red was used to detect changes in the proton equilibria following the binding of the inhibitor to the enzyme⁹⁷. The enzyme concentration was determined spectrophotometrically prior to dye addition using $\epsilon_{280\text{ nm}} = 50,000\text{ cm}^{-1} \times 1 \times \text{mol}^{-1}$.

Results

The data are summarized in Table 3 and on Fig. 19–23. All the inhibitors investigated were adsorbed to the enzyme, since both spectrum and the relaxation rate were dependent on as well the dye as the enzyme concentration, although the enzyme does not display a spectrum of its own in the visible range. In most cases, the change in dye-dye interaction, due to the binding of the monomer to the enzyme, was observed in the time range which can be reached with the temperature-jump method. Only in the cases of proflavine and acriflavine, the inhibitor-enzyme interaction was directly measured. The kinetic parameters, k_R and k_D , for the binding of these compounds to chymotrypsin, were determined. The equilibrium constant was measured by spectrophotometric titration. It was in reasonably agreement with other data, when a comparison was possible (Table 3). In some

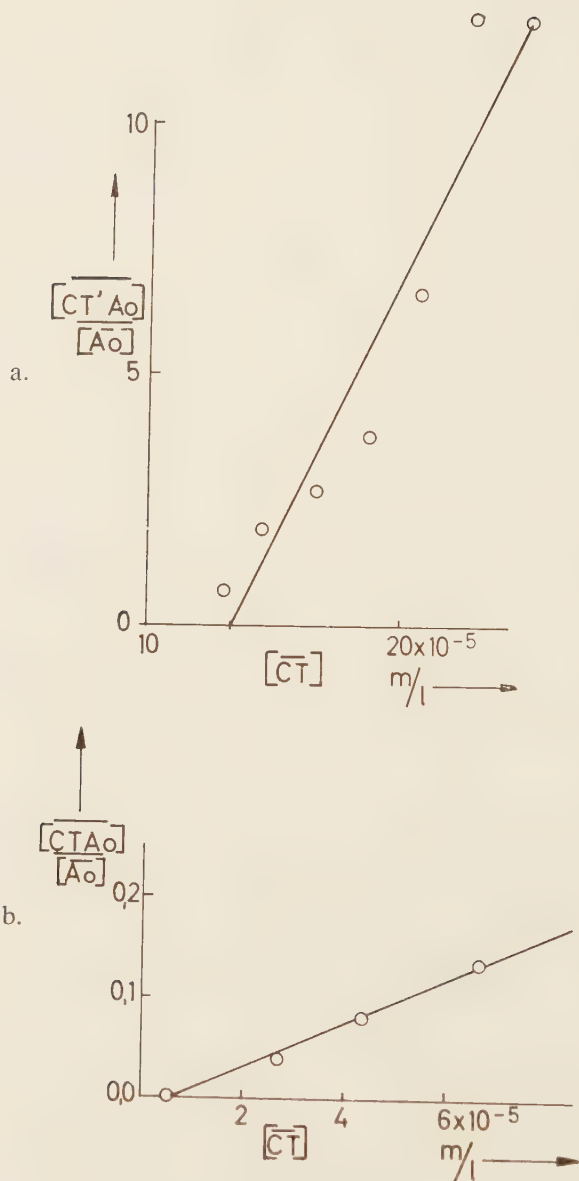


Figure 22

Spectrophotometric titration of acridine orange with CT at pH 6.7, 11° C and 0.10 *M* ionic strength (phosphate buffer).

a. Slope: $K_1 = \frac{[CT'AO]}{[CT]} \times \frac{[AO]}{[CTAO]}$;

b. Slope: $K_2 = \frac{[CT'AO]}{[CTAO]}$;

CTAO and CT'AO are isomers differing in the protein conformation.

Table 3

System	Ref.	T °C	pH	μ M	K_{spec} M $\times 10^{+4}$	k_R $1 \times M^{-1} \times \text{sec}^{-1}$ $\times 10^{-7}$	k_D sec^{-1}	Effects
PF + CT.....	This work	12	6.77	0.10	(3.14 ± 0.12)	(3.92 ± 0.71)	(2085 ± 335)	1
PF + CT.....	This work	12	9.2	0.10				2
PF + CT.....	This work	12	9.6	0.10				2
PF + CT.....	93	—	7.6	0.2	4.45			
PF + CT.....	91	25	6.3	—	2.5 ± 0.2			
PF + CT + ATEE.....	91	25	6.3	—	2.7 ± 0.3 ^a			
PF + CT + Ac-L-val-OMe	89	25	6.3	—	0.77 ± 0.24 ^a			
PF + PF.....	This work	12	6.7	0.1				Very fast
AF + CT.....	This work	12	6.7	0.1	2.25 ± 0.29	2.38 ± 0.54	2750 ± 445	1
AF + CT.....	89	25	6.3	0.2	1.25 ^a			2
AO + CT.....	This work	12	6.7	0.1	0.22			
CTAO ⇌ CT'AO.....	This work	12	6.7	0.1	48			
AO + AO.....	This work	12	6.7	0.1	1.22 ± 0.13	24.9	30910	1
AO + AO.....	98	14	6.0	0.1		80	20000	1
AO + AO.....	99	14	6.0	0.1	0.32		10 ⁵	
AO + polyphosphate.....	98							1
CT + 9-NH ₂ -acridine.....	This work	12	6.7	0.1				1
CT + acridine.....	This work, 90	12	6.7	0.1	0.3 ^a			1
CT + β-phenyl-propionate	This work	12	6.7	0.1				1
CT + N-Ac-3,5-DN-tyr...	This work	12	6.7	0.1				1
PF + indole.....	This work	12	6.7	0.1				1
PF + PF.....	100	25	4	0.05	0.2			

a Inhibition constant.

cases, an apparent discrepancy arose, because the kinetic method measured the actual binding step separately, while the equilibrium data provided overall constants which can include isomerizations of the enzyme or its complex with the inhibitor. Such apparent discrepancies can provide valuable indications about the detailed mechanism.

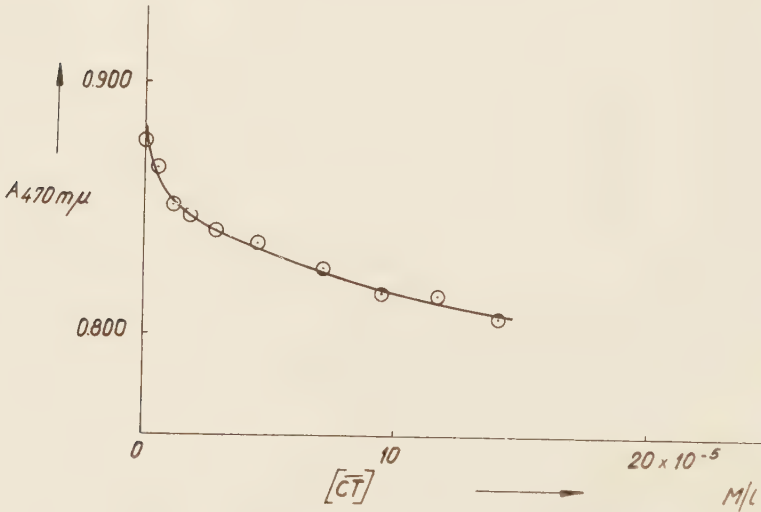


Fig. 23a

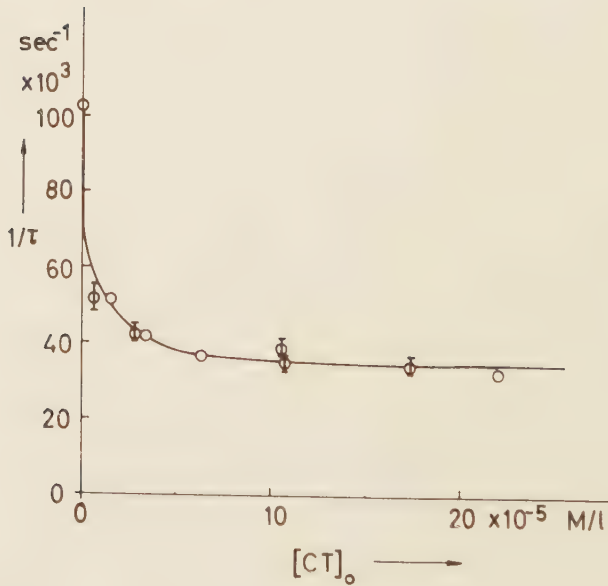


Fig. 23b

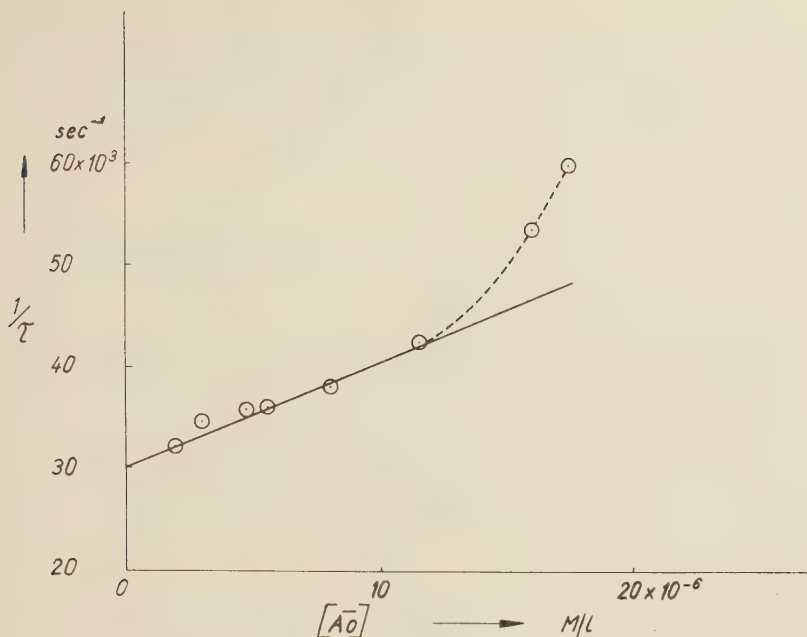


Fig. 23c

Figure 23

a. Suppression of the aggregation of acridine orange by CT. Spectrophotometric titration of acridine orange with CT at pH 6.7, 11° 3 C and 0.10 *M* ionic strength (phosphate buffer). The dimerization peak of acridine orange at 470 nm was followed. Reaction scheme: $(AO)_2 + 2CT \rightleftharpoons 2AO + 2CT \rightleftharpoons 2AOCT$.

b. The concentration dependence ($[CT_0]$) of the relaxation rate of dye dimerization.

c. The concentration dependence ($[AO]$) of the relaxation rate for dimerization of acridine orange at pH 6.7, 11° 3 C and 0.10 *M* ionic strength (phosphate buffer). Slope: 4 k_R . Intercept on the ordinate axis: k_D ; $[(AO)_2]/[AO]^2 = k_R/k_D = K$. The agreement between the kinetic data and the predicted rate law supports the hypothesis that acridine orange dimerization is observed. Higher aggregates occur at $[AO] > 1.2 \times 10^{-5}$ *M*.

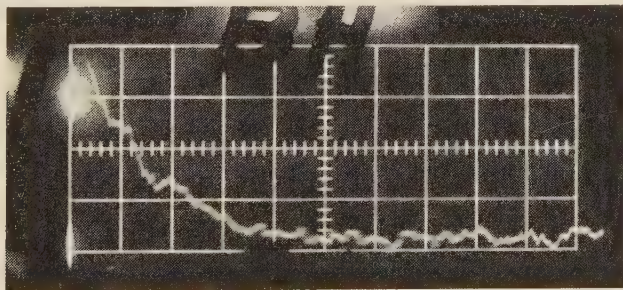


Figure 24

Relaxation curve. Interaction of α -chymotrypsin (1.06×10^{-5} *M*) with acridine orange (2.72×10^{-5} *M*) at pH 6.7 in 0.10 *M* phosphate buffer. Temperature jump: 5 to 12° C. Time sweep: 20 μ sec/cm. Ordinate: 20 mv/cm (band width: 1.3 Mc). 492 nm (band width: 14 nm).

The concentration dependence of the relaxation rate (Fig. 20–21) fitted simple binding kinetics at low and moderate concentrations (for acriflavine: below $2.5 \times 10^{-4} M$, for proflavine: at least below $1.4 \times 10^{-4} M$, but not exceeding $3.0 \times 10^{-4} M$). Only one relaxation effect was seen in this range.

Theory

The data were plotted as $1/\tau$ versus $(c_0 + c_1 - 2c_2)$ according to the equation:

$1/\tau = k_R(c_0 + c_1 - 2c_2) + k_D$, where

k_R = rate constant for recombination

k_D = rate constant for dissociation

c_0 = initial concentration of CT

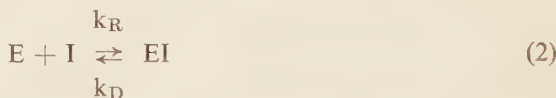
c_1 = initial concentration of dye

c_2 = equilibrium concentration of the dye-CT complex

$$2c_2 = (c_0 + c_1 + 1) - \sqrt{(c_0 + c_1 + 1)^2 - 4c_0c_1}$$

The positive sign before the square root represents a foreign root.

The activity coefficients were considered = 1. The reaction equation is:



If the perturbation by the temperature-jump is small, then the rate expression can be linearized and expressed as:

$$dx/dt + (1/\tau)x = (1/\tau)\bar{x} \quad (3)$$

where $x = c - c^0$ is the difference between the actual concentration, c , and a time-independent reference value, c^0 . Similarly, $\bar{x} = c - c^0$ is the difference between the equilibrium concentration, c , (temperature dependent and therefore, during the T-jump, also time dependent) and the reference concentration, c^0 . The rate equation is given by:

$$-dc_E/dt = -dc_I/dt = dc_{EI}/dt = k_R c_E c_I - k_D c_{EI} \quad (4)$$

By introduction of the definitions of x and $\bar{x}$ and $x_E = x_I$ (similarly: $\bar{x}_E = \bar{x}_I$), the equation becomes:

$$\begin{aligned} dc_E/dt &= dx_E/dt = k_D(\bar{c}_{EI} + x_{EI} - \bar{x}_{EI}) - k_R(\bar{c}_E + x_E - \bar{x}_E)(\bar{c}_I + x_I - \bar{x}_I) \\ &= k_D\bar{c}_{EI} - k_D(\bar{x}_{EI} - x_{EI}) - k_R(\bar{c}_E\bar{c}_I + k_R(\bar{c}_E + \bar{c}_I)(\bar{x}_E - x_E) + k_R(\bar{x}_E - x_E)^2 \end{aligned}$$

Since the equilibrium condition requires, that $k_R\bar{c}_E\bar{c}_I = k_D\bar{c}_{EI}$ and since the $(\bar{x} - x)$ -terms are assumed to be so small, that their squares can be ignored, the rate equation can be reduced to:

$$dx_E/dt + (k_D + k_R(\bar{c}_E + \bar{c}_I))x_E = (k_D + k_R(\bar{c}_E + \bar{c}_I))\bar{x}_E$$

By comparison with equation (3) one sees that:

$$1/\tau = (k_D + k_R(\bar{c}_E + \bar{c}_I)) = (k_D + k_R(c_0 + c_1 - 2c_2)) \quad (5)$$

Beer-Lambert's law was obeyed for the dye in the following concentration ranges:

Proflavine	$(0-5.0) \times 10^{-4} M$
Acriflavine	$(0-3.0) \times 10^{-4} M$
Acridine	$(0-5.0) \times 10^{-4} M$

The spectrophotometric titrations were carried out by adding aliquots of CT-stock solution to a solution of dye and buffer at the pH and concentrations which were used for the kinetic experiments. The bands of higher dye polymers were lying so close to the monomer band and the enzyme-dye-complex band, that great care was necessary to obtain a sufficient spectral resolution. This was for proflavine and acriflavine accomplished at 475 nm and for acridine orange at 500 nm.

Discussion

The relaxation curves measured were simple exponential functions. Therefore, the assumption, that the perturbation was small and only gave rise to a single relaxation effect at pH 6.7, was fulfilled. No relaxation effect was seen with chymotrypsin alone at pH 6.7. Hence, the observed, moderately fast effects must be ascribed to the enzyme-inhibitor interaction. In a solution only containing proflavine in buffer, a small and immeasurably fast relaxation effect reflecting the dye-dye interaction was seen, as expected. The enzyme did not loose activity due to the temperature jumps.

The studies of the concentration dependence of the relaxation rate, $1/\tau$, for the binding of proflavine to CT at pH-values near 9 (9.2 and 9.6), revealed the existence of an additional relaxation effect. The slower of the 2 effects is independent of the reactant concentrations. This is characteristic of an isomerization of the inhibitor-CT complex. The other effect has a marked concentration dependence which is typical of the complex formation step. A number of kinetic schemes involving ionization of the enzyme, enzyme-inhibitor complex formation and isomerization of such complexes were made and tested. Only schemes including the complex formation and the isomerization reaction could explain the observations.

Conclusions

The specificity study leads to the conclusion that the aromatic compounds used as models of specific substrates of chymotrypsin have a tendency to aggregate⁹⁹ (mostly dimerization) which often is strong. When the inhibitor to enzyme ratio

is not small, then this "substrate" aggregation must enter into the kinetic scheme of the catalytic reaction. It was spectrophotometrically and kinetically demonstrated that the enzyme depolymerizes the aggregate of the "substrate" molecules. This is particularly spectacular in the case of acridine orange. The dye-dye interaction is slower than, or has a rate comparable to, that of the enzyme-dye reaction, except in the cases where the acridine nucleus carries a free amino group in the 3 and 6 positions (proflavine and acriflavine). When these amino groups are alkylated, each with 2 methyl groups (acridine orange), then the dye-dye interaction again prevails in the reaction kinetics. Besides, both the spectrophotometric titration and the kinetics show that the binding of acridine orange to chymotrypsin is followed by an isomerization of the complex.

The fit of the data for the titration of proflavine with chymotrypsin to a simple hyperbolic relationship confirms that the interaction is stoichiometric (1:1). Since the dimerization constant for proflavine is known, it is possible to conclude that it is the monomeric form of proflavine which binds to the enzyme.

The observation that the alkylation of proflavine to acridine orange only leads to a minor change in the spectrum indicates that the aggregation at least partly is hydrophobic in nature. A contribution from a charge transfer is unlikely to be important. It is known that the biological effect of acridine derivatives strongly depends on the presence of the charge on the ring-N. Therefore, ionic bonding very likely is playing a role, as well in aggregation reactions of the inhibitors, as in their complex formation with enzymes as chymotrypsin and trypsin.

The multiple relaxation effects which appeared with proflavine and acriflavine at pH 9.2 and 9.6, and with acridine orange even at pH 6.7, could only be accounted for by proposing kinetic scheme which included 2 consecutive steps, a binding reaction followed by an intramolecular rearrangement. This evidence strongly favours the hypothesis based on previous static data that the catalytic action of chymotrypsin and trypsin is accompanied by conformational changes in the enzyme.

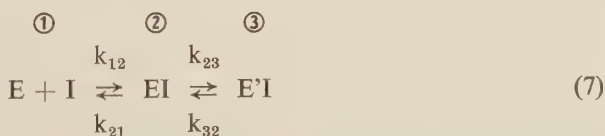
3.1.3. Evaluation of rate constants for elementary steps

Previous studies of the static properties of chymotrypsin and trypsin and of isolated enzyme-substrate (or enzyme-inhibitor) complexes led to the development of the hypothesis that the catalytic action of these (and some other) enzymes is accompanied by conformational changes in the complex^{4, 74, 62, 43}.

Since the changes in the physical properties and the chemical reactivity of the catalyst were very marked, it was at first expected that the configurational changes were quite large. However, subsequent optical rotatory dispersion studies in the far ultraviolet range⁶⁰ and x-ray crystallographic analysis¹⁰¹ showed that the changes in the overall conformation were detectable, but not extensive. Hence, the rearrangement must be rather localized, but critical, and it is probably affecting the groups which are essential to the catalytic mechanism almost directly.

These kinetic studies were designed to attempt the detection of the operation of an isomerization reaction of an enzyme-substrate complex in the catalytic mechanism by a new and independent approach. A chemical relaxation technique was chosen because it permits the detection of all elementary steps which can come in question, although they are fast. The evaluation of the individual rate constants and properties, such as activation parameters, proved in many cases to be possible.

In the first series of experiments, the specificity of the interaction was investigated using a number of acridine derivatives and other substances which are known to bind in a stoichiometric fashion to chymotrypsin and trypsin and to be competitive inhibitors of these enzymes. Proflavine was chosen for a more detailed study of the pH range 8–10 where the enzymes have their maximal activity and where a sequence of 2 consecutive steps, a bimolecular followed by an unimolecular step, was found. Proflavine was chosen as a substrate model because the position of its chromophore is convenient and because the dimerization reaction of the dye for many purposes may be ignored. The good fit of the data to the scheme:



permits the exclusion of a simple binding reaction and strongly supports the hypothesis of the occurrence of an isomerization reaction in the catalytic sequence.

The possibility that the data reflect a transfer of the inhibitor from one site to another on the enzyme can be excluded by the kinetics of the reaction at low reactant concentration.

Experimental

Materials: Chymotrypsin was a 3 times crystallized, saltfree product from Worthington Biochemical Corporation (CDI). The activity was checked by the method of Schwert et al. with N-acetyl-L-tyrosine ethyl ester as substrate⁹⁴. Trypsin was a twice recrystallized, lyophilized preparation (TRL 6260) from the same company. Proflavine was purchased as hemisulfate from British Drug House and twice recrystallized from aqueous methanol. After purification, the substance was chromatographically homogeneous. The buffer substances, tris-(hydroxymethyl)-amino-methane, potassium phosphate, borax, potassium carbonate and β -phenyl-propionate, were analytic reagents purchased from Merck. All solutions were prepared with redistilled, degassed water.

Instrumental: The relaxation experiments were carried out by the temperature-jump method as described by Eigen and DeMayer⁹⁶. The initial temperature was 4.5° C and the temperature change was about 7 degrees. The wavelength 404 nm was selected with an interference filter (Bandwidth: 14 nm) from Jena Glaswerke (Schott & Gen.). The absorbancy changes were recorded on a Textronix oscilloscope and the curve was photographed with a Robot camera.

The pH was measured at 12° C with a Radiometer 22r pH-meter after calibration with standard (NBS) buffers⁹⁵. The spectra were recorded at 12° C (except the CT assays which were made at room temperature) with a Beckman DK2A ratio-recording spectrophotometer using thermostated cuvettes.

The concentration of the enzyme was spectrophotometrically determined prior to the addition of the inhibitor using the extinction coefficients: For chymotrypsin: $2.00 \text{ cm}^{-1} \times 1 \times \text{mg}^{-1}$ at 280 nm; for trypsin: $1.71 \text{ cm}^{-1} \times 1 \times \text{mg}^{-1}$ at 282 nm¹⁰². The molecular weight of chymotrypsin was taken as 25000 and that of trypsin as 23100. The initial concentration of proflavine was determined by weighing and checked spectrophotometrically. Stoichiometric quantities (1:1) of the reactants were used throughout. The ionic strength of the solutions was 0.10 *M*. The main contributor to the ionic strength was the buffer (phosphate at pH 6.7, tris at pH 8.46 and 9.18, borate at pH 9.6 and carbonate at pH 10.3). At pH 10.3 the ionization of the proflavine is so strong that the dye had to be substituted with another competitive inhibitor, β -phenyl-propionate. Since this substance does not absorb in the visible range, the reaction was followed by use of a protolytic indicator, phenolphthalein, which responds to the changes in proton equilibria following the isomerization. The coupling between the elementary steps ensures that this indicator records the total system.

Proflavine is known to dimerize and deviations from Lambert-Beer's law were found at concentrations above $1.2 \times 10^{-4} \text{ M}$ at pH 7.6, but the equilibration is very fast and the concentration of the dye is kept so low in the rate experiments that the dimerization reaction does not interfere significantly. Only the charged form of PF binds to the enzymes.

Theory

The linearized rate equation for a multiple-step system is:

$$dx_i/dt + \sum_k a_{ik} x_k = \sum_k a_{ik} \bar{x}_k \quad (8)$$

where $x_i = c_i - c_i^0$ is the difference between the actual concentration c_i and a time-independent reference value c_i^0 of component *i*. Similarly, $\bar{x}_i = c_i - c_i^0$ is the difference between the equilibrium concentration c_i (temperature dependent and therefore during the T-jump also time dependent) and c_i^0 . The figure *k* is

an index for a kinetic state. The assumption that the perturbation is small, i.e. that $(\bar{x}_i - x_i) \leq c_i$ is good, since the relaxation curves are simple exponential decay functions. Therefore, the linearization of the rate equations is permissible. The a_{ik} factor represents coefficients containing rate constants and equilibrium concentrations, according to the stoichiometry of the reactions. There are n rate equations if there are n representative variables, x_i , i.e. n steps. For the two-step reaction scheme which here is discussed (7), the linearized rate equations are:

$$\begin{aligned} dx_1/dt = & -k'_{12}x_1 + k_{21}x_2 = -k_{12}c_Ec_I + k_{21}c_{EI} = \\ & -k_{12}(c_E - x'_1)(c_I - x'_1) + k_{21}(c_{EI} + x'_2) \end{aligned} \quad (9)$$

$$dx_2/dt = k'_{12}x_1 - (k_{21} + k_{23})x_2 + k_{32}x_3 \quad (10)$$

$$dx_3/dt = k_{23}x_2 - k_{32}x_3 \quad (11)$$

where $k'_{12} = k_{12}(c_E + c_I)$,

$$x_1 = c_E - c_E^0 = c_I - c_I^0$$

$$x_2 = c_{EI} - c_{EI}^0$$

$$x_3 = c_{E'I} - c_{E'I}^0$$

$$c = x + c^0 = x + c - \bar{x} = c - (\bar{x} - x) = c - x'$$

Terms containing squares of $x'_i = \bar{x}_i - x_i$ are ignored, because they are very small. All terms only containing equilibrium concentrations cancel due to the equilibrium condition (see p. 52). The law of mass conservation permits the reduction of the number of simultaneous differential equations from 3 to 2. The characteristic equation for the system is in determinantal form:

$$\begin{vmatrix} (a_{11} - b) & a_{12} \\ a_{21} & (a_{22} - b) \end{vmatrix} = (a_{11} - b)(a_{22} - b) - a_{12}a_{21} = 0 \quad (12)$$

where b_{ii} , the eigenvalues, are the reciprocal relaxation times:

$$b_{1,2} = -1/\tau_{1,2} \quad (13)$$

Solution of the quadratic equation gives: $b_{1,2} = (L \pm \sqrt{L^2 - 4M})/2$

where $L = a_{11} + a_{22} = k'_{12} + k_{21} + k_{23} + k_{32} = b_1 + b_2$ and (14)

$$M = a_{11}a_{22} - a_{12}a_{21} = k'_{12}(k_{23} + k_{32}) + k_{21}k_{32} = b_1b_2 \quad (15)$$

Hence, a plot of the sum of the observed relaxation rates, $(1/\tau)$, as a function of the sum of the equilibrium concentrations of E and I gives a straight line (Fig. 26) with the slope k_{12} and the intercept: $(k_{21} + k_{23} + k_{32})$. Similarly, a plot of the product of the reciprocal relaxation times, $= M$ (Fig. 27), as a function of

the sum of the equilibrium concentrations of E and I, yields as slope $k_{12}(k_{23} + k_{32})$ and as intercept on the ordinate axis: $k_{21}k_{32}$. All the 4 rate constants of the elementary reactions can be calculated from this information.

Results

Two relaxation effects were observed at pH 8.5-10.3, but only one at pH 6.7. The dependence of each of the effects on the sum of the equilibrium concentrations of chymotrypsin and proflavine is shown in Fig. 25 for the data at pH 9.18. Before the evaluation of the data, it was necessary to assume a value for the equilibrium constants for the complex formation and the isomerization in order to calculate the equilibrium concentrations of the reactants. It was at first assumed that the equilibrium concentrations could be considered equal to the initial concentrations of enzyme and inhibitor, i.e. that the equilibria were displaced towards dissociation. With this assumption, the rate laws were excellently obeyed. However, when the actual equilibrium constants calculated as ratios between the rate constants were introduced, it was found that the concentrations of the intermediates EI and E'I appeared to be appreciable. Only the expansion of the scheme with a fast preequilibrium involving an enzyme transition could resolve the apparent discrepancy. This enzyme isomerization was demonstrated at pH 9 in the absence of the inhibitor using a temperature-jump apparatus with polarization optics. The fast change in optical rotation revealed a relaxation

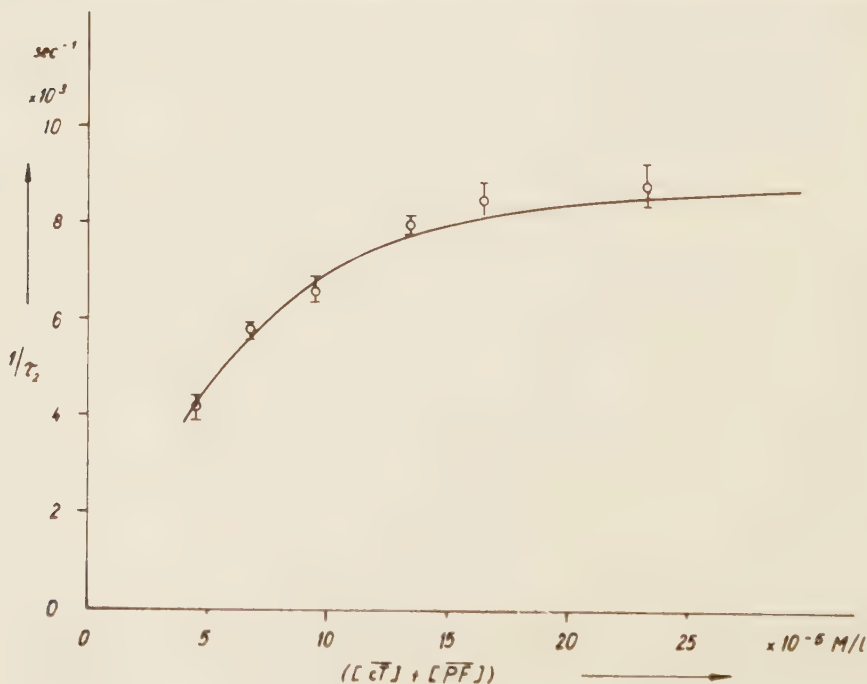


Fig. 25a

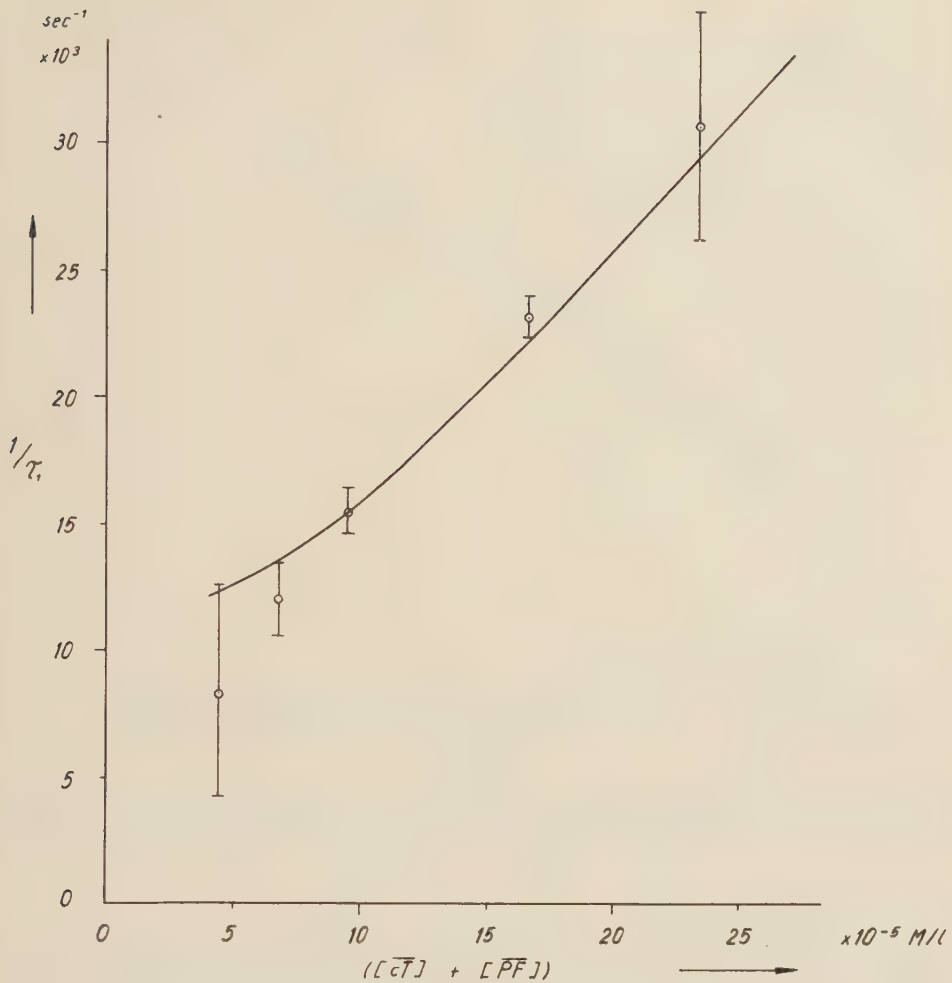


Fig. 25b

Figure 25

The concentration dependence of the relaxation effects for the system: Chymotrypsin + proflavine at pH 9.18, 12° C and 0.10 *M* ionic strength (tris buffer). The curves are calculated from the parameters in Table 4. These were determined by linear regression. a. The slow effect which mostly reflects the isomerization of the enzyme-inhibitor complex. b. The fast effect which mostly is determined by the collision step. The deviation at low concentration is probably due to the preequilibrated CT-transition: $CT_1 \rightleftharpoons CT$.

effect which was located too far from the other effects to couple significantly with the collision step and the conformational change of the inhibitor-enzyme complex. This explanation is plausible, if only one of the 2 chymotrypsin isomers binds proflavine and if the CT-isomer, which does not bind the inhibitor appreciably, prevails before the perturbation. Static and dynamic data obtained by Lumry⁴³ and others indicate, that this is correct.

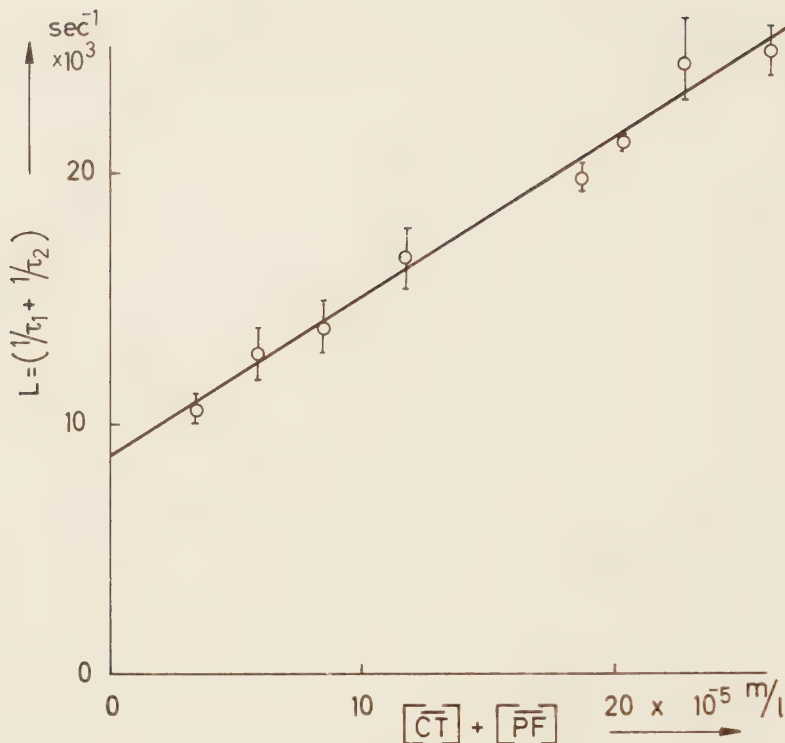


Figure 26

The concentration dependence of the function $L (= 1/\tau_1 + 1/\tau_2)$ for the system: CT + PF at pH 8.46 and 12° C. The line is calculated by regression.

The protolytic dissociation of proflavine was found by spectrophotometric titration to have a pK' -value of 10.07 ± 0.03 at 12° 7 C. Since the basic form of the dye is insoluble and since proflavine is biologically inactive when the charge is removed from the nitrogen atom of the acridine ring system, it was assumed that only the acidic form of the dye bound to the enzymes. Therefore, a small correction in the concentration term had to be introduced in the alkaline end of the pH range investigated.

The correctness of the treatment of the data was confirmed by the reasonable agreement which was found between the kinetically determined equilibrium constant and that determined by inhibition studies with real substrates, by equilibrium dialysis and by spectrophotometric titration. Smaller deviations arose from the fact that all static methods yielded overall equilibrium constants, while the relaxation kinetics gave the parameters referring to elementary steps. A summary of the data is presented in Table 4 and illustrated on Fig. 28. Since the results obtained with the other competitive inhibitor, β -phenyl-propionate, agreed with those for proflavine, the kinetic scheme proposed seems to be general.

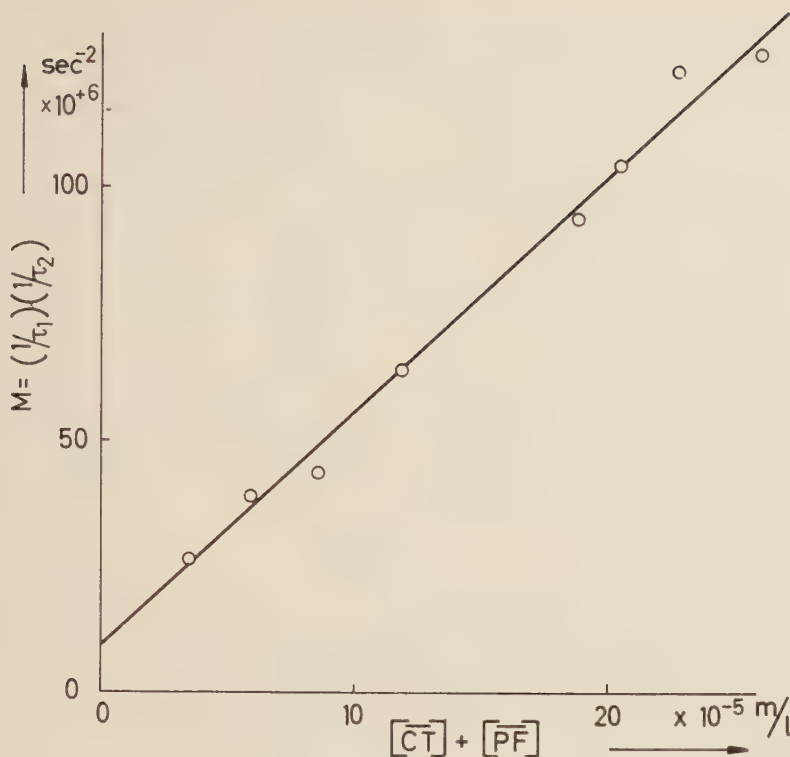


Figure 27

The concentration dependence of the function $M (= (1/\tau_1) \times (1/\tau_2))$ for the same system as in Fig. 26. Line: Regression line.

Table 4^a

pH	k_{12}^{obs} $M^{-1} \times 1$ $\times \text{sec}^{-1} \times 10^{-8}$	k_{21}^{obs} sec^{-1}	k_{23}^{obs} sec^{-1}	k_{32}^{obs} sec^{-1}	K_I^b $1 \times M^{-1}$ $\times 10^{-5}$
6.69	(0.39 ± 0.07)	2085 ± 335	0	0	0.19 ± 0.04
8.42	(0.63 ± 0.03)	1230 ± 640	525 ± 350	7045 ± 350	0.51 ± 0.25
9.18	(1.14 ± 0.02)	2150 ± 250	7300 ± 700	2000 ± 300	0.53 ± 0.03
7.9 ^c					0.25 ± 0.02

a 12°C, 0.10 M ionic strength and $[CT]/[PF] = 1$.

b $K_I = k_{12}^{\text{obs}}/k_{21}^{\text{obs}}$.

c 25°C.

Discussion

The kinetic data for the interaction of chymotrypsin with the competitive inhibitor, proflavine, fit at each pH in the main range, a simple 2-step scheme con-

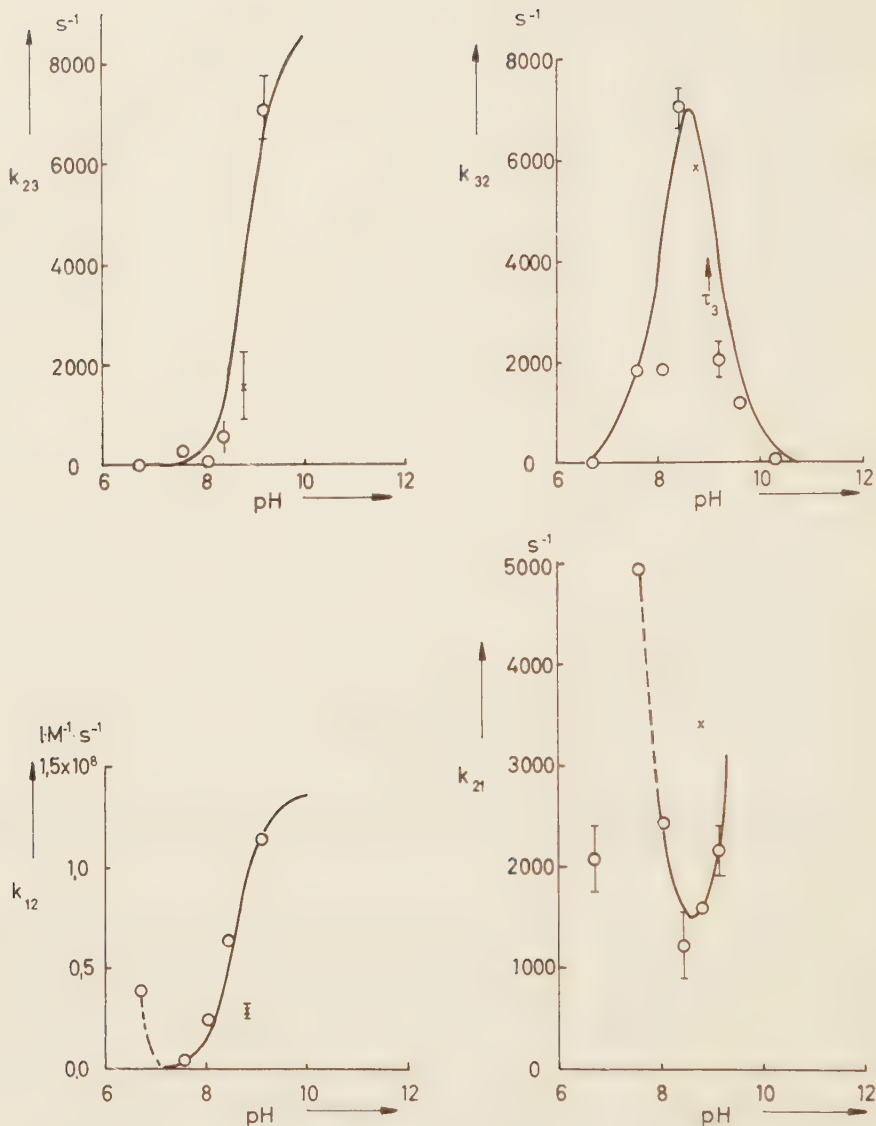


Figure 28

The pH-dependencies of the observed rate constants: k_{12} , k_{21} , k_{23} and k_{32} at 12°C and 0.10 M ionic strength. The point at pH 10.3 represents the interaction of CT with β -phenyl-propionate (indicator: phenolphthalein). The symbols: x represent the interaction of trypsin with proflavine. The arrow at pH 9 indicates the location of the relaxation effect for the fast (ca. 10 μ sec) CT-isomerization ($CT_1 \rightleftharpoons CT$). This was observed at 365 nm using a T-jump apparatus with polarization optics. The errors shown are standard deviations. The curves are theoretical curves calculated from the data in Table 5 using the equations discussed in the text.

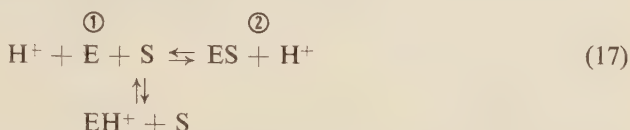
sisting of 2 consecutive steps, a bimolecular collision followed by an isomerization of the enzyme-inhibitor complex. The pH-dependence of each of the rate constants shows, that the main reaction is coupled to a number of rapid proton transfer reactions. This is illustrated in Fig. 28. A detailed analysis of the four rate constants gave the following result:

k_{12} : The pH-dependence of k_{12} is sigmoidal. A theoretical curve which fits these data should have the form:

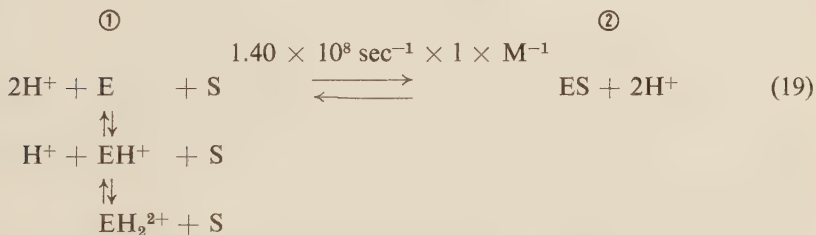
$$\begin{aligned} \text{for 1 proton: } k_{12 \text{ obs}} &= k_{12}/(1 + [\bar{H}^+]/K_a^E) = \\ & k_{12}[\bar{E}]/([\bar{E}] + [\bar{E}\bar{H}]) = k_{12}[\bar{E}]/\Sigma[E] \end{aligned} \quad (16)$$

$$\Sigma[E] = [\bar{E}] + [\bar{E}\bar{H}]$$

where k_{12} is the rate constant for the elementary step and K_a the protolytic dissociation constant. This corresponds to the scheme:



$$\begin{aligned} \text{for 2 protons: } k_{12 \text{ obs}} &= k_{12}/(1 + [\bar{H}^+]/K_{a1}^E + [\bar{H}^+]^2/(K_{a1}^E K_{a2}^E)) = \\ & k_{12}/(1 + [\bar{E}\bar{H}]/[\bar{E}] + ([\bar{E}\bar{H}_2]/[\bar{E}])) = \\ & k_{12}/(1 + [\bar{E}\bar{H}]/[\bar{E}] + ([\bar{E}\bar{H}]/[\bar{E}])([\bar{E}\bar{H}_2]/[\bar{E}\bar{H}])) = \\ & ([\bar{E}]/\Sigma[E])k_{12} \end{aligned} \quad (18)$$



Since the inflection point of the sigmoidal curve corresponds to a pH of about 8.6, only a limited number of classes of ionizing groups can come in question for chymotrypsin. This value is typical for α -amino and sulfhydryl groups, but could also be due to anomalous ϵ -amino, phenol or even imidazol groups. However, sulfhydryl groups are not present in chymotrypsin and the two tyrosyl sidechains ionize identically in the free enzyme and in the isolated enzyme-inhibitor compound, diisopropylphosphoryl-chymotrypsin⁷¹. Therefore, it should be permissible to eliminate these residues. Hess et al.⁴⁶ have found that δ -chymotrypsin shows the normal catalytic properties when the ϵ -amino groups and the A-chain N-terminus are masked by acetylation. If, however, the only remaining α -amino group is blocked by reacylation, then the enzyme loses all its activity. The activity returns (80%) after specific hydrolysis of the ester groups of the B-terminus. It is therefore highly probable that one or both of the N-terminal α -amino

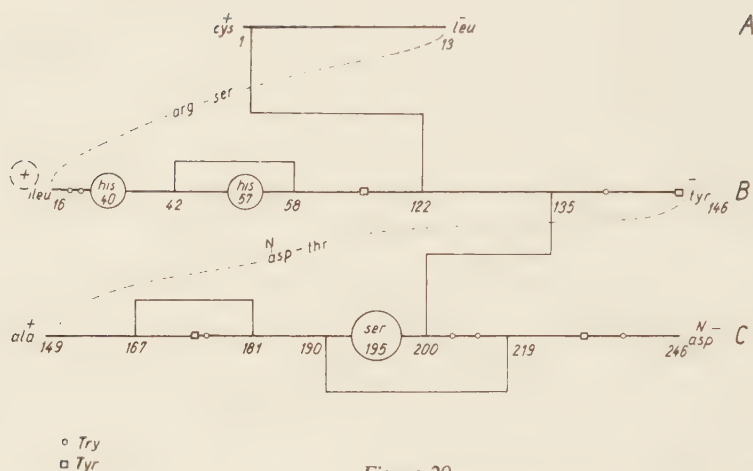
α -Chymotrypsin

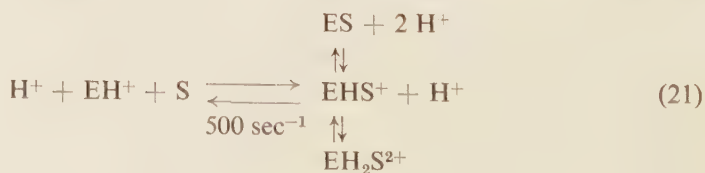
Figure 29

The structure of α -chymotrypsin. The peptide chains are marked with A, B and C. The aminoacid residues which are essential to catalysis are marked by a circle. The dotted lines indicate the connections in the zymogen. The two dipeptides, arg-ser and N-asg-thr are removed in the course of the activation reaction (tryptic cleavage followed by autolysis).

groups are responsible for the pH-dependence of k_{12} (See Fig. 29). The theoretical curve for 2 α -amino groups of identical pK' -value (8.60 ± 0.05) fit the data better than only one group of this pK' -value, but the significance of the difference was only moderate considering the experimental error. Two groups of $pK' = 8.50$ also fitted the data poorer than 2 with $pK' = 8.60$. The value of $(1.40 \pm 0.05)10^8 \text{ l} \times \text{Mol}^{-1} \times \text{sec}^{-1}$ for k_{12} gave a good fit of the data. Similarly, it was possible to estimate the three remaining rate constants:

k_{21} : The pH-dependence of this rate constant was bellshaped with a minimum near pH 8.6. In this case, at least 2 ionizing groups with a pK' near 8.6 must have been operating. Very likely, it was the same groups as in the case of k_{12} , but here the data required a mechanism which could be described by an equation of the form:

$$k_{21 \text{ obs}} = k_{21}(1 + [\text{H}^+]/K_{a2}^{\text{ES}} + K_{a1}^{\text{ES}}/[\text{H}^+]) = k_{21}(1 + [\text{EH}_2\text{S}]/[\text{EHS}] + [\text{ES}]/[\text{EHS}]) = k_{21}/([\text{EHS}]/\Sigma[\text{ES}]), \text{ where } \Sigma[\text{ES}] = [\text{ES}] + [\text{EHS}] + [\text{EH}_2\text{S}] \text{ i.e. EHS was the important species. This corresponds to the scheme:} \quad (20)$$



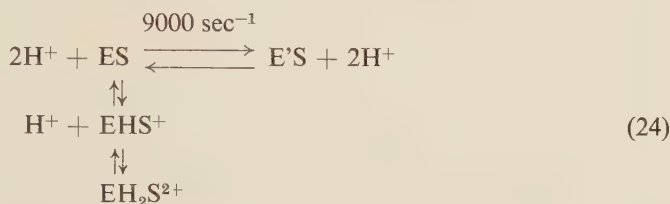
The values $pK'_{a1} = pK'_{a2} = 8.60 \pm 0.05$ and $k_{21} = 500 \pm 50 \text{ sec}^{-1}$ determined a theoretical curve which fitted the experimental data well. The requirement was:

$$[\bar{H}^+_{\min}] = \sqrt{K_{a1}K_{a2}} \quad (22)$$

k_{23} : In the isomerization steps, k_{23} fitted the equation:

$$k_{23 \text{ obs}} = k_{23}/(1 + [\bar{H}^+]/K_{a1}^{\text{ES}} + [\bar{H}^+]^2/K_{a1}^{\text{ES}}K_{a2}^{\text{ES}}) = k_{23}[\bar{E}\bar{S}]/\Sigma[\text{ES}] \quad (23)$$

The curve was sigmoidal and ES was required for the reaction onwards. The corresponding mechanism was:



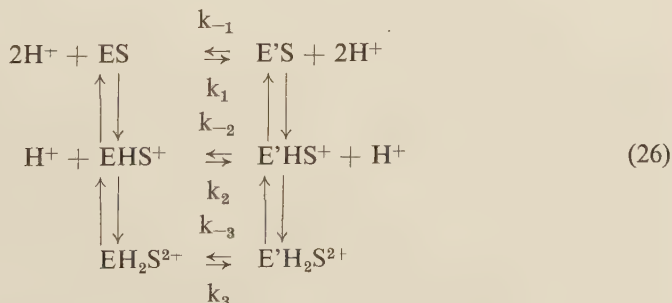
i.e. two consecutive proton equilibria were coupled to the isomerization. The curve could not be fitted with one pK' -value alone, but with two groups of identical pK' , i.e. no direct initial interaction, it could be done. The best fitting values were:

$$pK'_{a1} = pK'_{a2} = 8.70 \pm 0.08 \text{ and } k_{23} = 9000 \pm 500 \text{ sec}^{-1}.$$

k_{32} : In the reverse direction, the observed data satisfied the equation:

$$k_{32 \text{ obs}} = k_{32}/(1 + [\bar{H}^+]/K_{a1}^{\text{E}'\text{S}} + K_{a2}^{\text{E}'\text{S}}/[\bar{H}^+]) = k_{32}([\bar{E}\bar{H}\bar{S}]/\Sigma[\text{ES}]) \quad (25)$$

In this case, EHS was the important species and there were two proton pre-equilibria directly coupled to EHS. Therefore, the simpler of the possible schemes was:



where the path in the middle is prevailing.

The complete expression was:

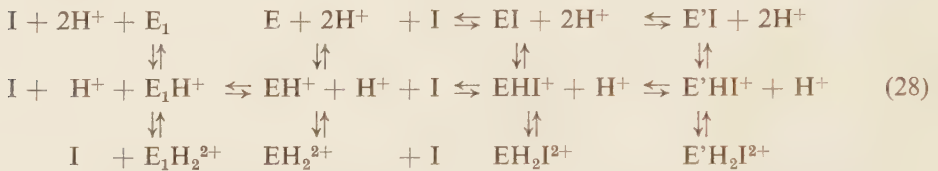
$$k_{32 \text{ obs}} = \frac{k_1 K_{a1} K_{a2} + k_2 [\bar{H}^+] K_{a2} + k_3 [\bar{H}^+]^2}{K_{a1} K_{a2} + [\bar{H}^+] K_{a2} + [\bar{H}^+]^2}, \text{ where} \quad (27)$$

$k_1 = k_3$ due to the symmetry of the curve and

$k_2 = 2.1 \times 10^4 - 2k_1 \cong 2.1 \times 10^4 \text{ sec}^{-1}$ assuming $K_{a1} = 10^{-8.6}$.

The experimental values agreed well with the theoretical curve determined by the values: $pK'_{a1} = pK'_{a2} = 8.60 \pm 0.05$, (the requirement: $\sqrt{K_1 K_2} = [\bar{H}^+]_{\text{max}}]$) and $k_{32} = (2.10 \pm 0.10) \times 10^4 \text{ sec}^{-1}$.

When all the information was combined (Table 5), then the following scheme appeared:



The first assumption was: $E_1 = E'$.

Table 5^a

Reaction	k_{12} $s^{-1} \times 1$ $\times M^{-1}$ $+ 10^{-8}$	k_{21} sec^{-1}	k_{23} sec^{-1}	k_{32} sec^{-1} $\times 10^{-4}$	K_I $1 \times M^{-1}$ $\times 10^{-5}$	K_{II}	pK_{a1} $= pK_{a2}$
1 $\rightarrow$ 2.....	1.40 $\pm$ 0.05				2.8 $\pm$ 0.3		8.60 $\pm$ 0.05
2 $\rightarrow$ 1.....		500 $\pm$ 50					8.60 $\pm$ 0.05
2 $\rightarrow$ 3.....			9000 $\pm$ 500			0.43 $\pm$ 0.02	8.70 $\pm$ 0.08
3 $\rightarrow$ 2.....				2.10 $\pm$ 0.10			8.60 $\pm$ 0.05

a 12°C, 0.10 M ionic strength, $K_I = k_{12}/k_{21}$, $K_{II} = k_{23}/k_{32}$.

This mechanism satisfied all data which so far are known for the system. No more than three relaxation effects are expected, since the buffer concentration is sufficiently high to make the proton transfer reactions occur much faster than the binding and isomerization steps. In the pH range near 9, chymotrypsin exists in two conformations. Since only one of these (appreciably) reacts with the substrate model, this isomerization preequilibrium, which solely concerns the enzyme, must be included in the kinetic scheme. It is probable that all the reactions seen in the chymotrypsin-proflavine system are controlled by the state of ionization of the N-termini of the B- and C-chains of chymotrypsin (iso-leucine in the B-chain and alanine in the C-chain).

The pK' -values of the termini slightly change as the result of the binding process, but they are hardly affected by the conformational change released by the inhibitor. The latter finding is surprising in view of the results with substrates as diisopropylfluorophosphate^{4, 74}, nitrophenyl-acetate⁴ or cinnamoyl-imidazole⁴³ and may only apply to inhibitors such as proflavine. It remains to be investigated, whether real substrates induce a second, consecutive conformational change or a single isomerization. A pK -displacement of more than one pK -unit was found for the formation of diisopropylphosphoryl-chymotrypsin. This indicates that the structural changes in this case are unusually pronounced.

It is now possible to calculate the equilibrium constants for the single steps from the rate constants:

$$K_I = [\bar{E}\bar{S}]/([\bar{E}] \times [\bar{S}]) = k_{12}/k_{21} = 1.40 \times 10^8/500 = 2.8 \times 10^5 \text{ l/Mol.}$$

Bernhard found: $(7.7 \pm 0.2) \times 10^5 \text{ l/Mol}$ at pH 6.3 using acetyl-L-valine methyl ester and proflavine under steady-state conditions⁹¹. Similar values were found in binding studies by the equilibrium dialysis method. For the inhibitor-induced conformational change in chymotrypsin, the following value was found for the equilibrium constant:

$$K_{II} = k_{23}/k_{32} = 9000/21000 = 0.43$$

A value near 1 was expected, since equilibria with constants, which are more than one order of magnitude removed from this value, are unlikely to exert a delicate control of the catalytic mechanism.

Preliminary studies with trypsin and proflavine were made to see if the extensive similarity between chymotrypsin and trypsin also is reflected in the pH-dependence of the rate constants. Gutfreund et al. found that proflavine also is a specific and competitive inhibitor of trypsin^{63, 64}. Only a single pH-value was tested, but in that case (symbol x in Fig. 28), the k_{23} and k_{32} fell quite close to the curves for chymotrypsin. For k_{12} and k_{21} , the agreement was poorer. Trypsin followed at the chosen pH-value (8.8) a scheme consisting of two consecutive steps, a bimolecular complex formation followed by an isomerization of the complex. Therefore, the two enzymes may follow the same general mechanism in the first steps of the catalytic process.

The rate constants of the elementary steps for the chymotrypsin-proflavine interaction and the limiting values for collision-dissociation reactions involving proteins ($10^9 \text{ l} \times \text{Mol}^{-1} \times \text{sec}^{-1}$ and 10^9 sec^{-1} according to Eigen and Hammes¹⁰³) were used to estimate the free energies of activation. If these are combined with the thermodynamic data of Lumry⁴³ and others, then it is possible to construct a potential energy diagram. A sketch of such a diagram has been made, but further interpretation should preferably await the confirmation of the estimates by direct measurement of the temperature dependence of the rate constants of the elementary steps.

Conclusions

1. It was shown that the kinetics of the interaction of α -chymotrypsin with the specific, competitive inhibitor, proflavine, can be described by two discrete, consecutive steps: a bimolecular reaction followed by a unimolecular one:



All the rate constants, k_{12} , k_{21} , k_{23} and k_{32} , could be evaluated without any other assumption than that the reaction was displaced towards left before the temperature rise. This view was supported by direct measurement of the pre-equilibrium: $\text{CT}_1 \rightleftharpoons \text{CT}$ at pH 9 in the absence of proflavine using a temperature-jump apparatus with polarization optics. The kinetically determined equilibrium constants were in reasonable agreement with those obtained by spectrophotometric titration and equilibrium dialysis and from inhibition constants.

2. The pH-dependencies of the rate constants were determined at pH 6.7–10.3 and kinetic equations, which fit the experimental data, were developed.

3. The rate laws established for the single steps and some information from temperature-jump experiments, spectrophotometric titration and equilibrium dialysis were used to assemble a kinetic scheme for the interactions of chymotrypsin with the competitive inhibitor.

4. The interaction of proflavine with trypsin at pH 8.8 appeared to follow the same pattern as those found for the related enzyme, chymotrypsin, under the same conditions.

3.2. GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

3.2.1. Changes in the optical rotatory dispersion of the apoenzyme upon formation of the NAD-holoenzyme and an enzyme-substrate-intermediate

Although most evidence is available for the occurrence of conformational changes in transferases, the view that the phenomenon is very general is gaining acceptance. This work was designed to provide information about the symmetry of a typical oxidation-reduction enzyme, its protein part per se, as well as its complexes with a cofactor (NAD) and a substrate (G3P). The latter is an intermediate in the catalytic mechanism. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was chosen because of its key position in glycolysis and because many of its properties already are quite well established¹⁰⁴⁻¹⁰⁹. Studies of G3PDH have a special interest because it is an enzyme consisting of subunits (4 identical or almost identical chains). Some enzymes of this form are according to the hypothesis of Wyman, Monod¹¹ and others subject to allosteric control i.e. the tetramer exists in two conformations whose interconversion is influenced by a cofactor, a reaction product or a substrate. In this case the so-called allosteric effector is NAD. The work presented here shows that the symmetry, as measured by the optical rotatory dispersion parameters, clearly is changed when NAD is added to the apo-enzyme. The finding has later been confirmed by Listowsky et al.¹¹⁰. Kirschner, Bittman and Voigt¹² recently discovered that the titration curve of G3PDH with NAD at pH 9.0 was hyperbolic at 10 and 20° C, but clearly sigmoidal at 40° C. This is also true at pH 8.5 which represents an acidity maximum of the pH-profile of the activity. Such results cannot be explained by simple binding, but they fit the allosteric hypotheses¹¹ well. Kirschner et al.¹² subsequently demonstrated that the system displayed 3 relaxation effects and that the concentration dependence of the relaxation times accurately fitted the Wyman-Monod model (see p. 93), while a system of uncoupled binding equilibria as the Adair scheme¹¹¹, which contains four independent binding reactions, can be eliminated. These conformational changes induced by NAD precede, and may be independent of, rearrangements released by the actual substrate, G3P. Therefore, two true intermediates in the catalytic process were prepared and their optical rotatory dispersion parameters were compared with those of the apo- and the holo-enzymes.

Hvidt and Kägi^{55, 56} have found changes in the rate of deuterium-hydrogen exchange in yeast alcohol dehydrogenase on addition of NAD. This phenomenon, which was studied by infrared spectrophotometry, also indicates the occurrence of conformational changes in dehydrogenases following substrate binding.

Boyer^{112, 113} already has reported a change in the specific rotation of G3PDH after addition of NAD, but optical rotatory dispersion (O.R.D.) measurements are required to clarify whether the origin of the change in optical rotation is a change in the amplitude of a Cotton effect arising from helical structures, solvation, cofactor-apoenzyme complexes, metal ion-enzyme complexes or a source which so far has escaped attention. Observations similar to those of Boyer were made by Elödi and Szabolcsi¹¹⁴.

Experimental

Materials: A suspension of crystals of yeast G3PDH in a saturated solution of the enzyme containing NAD (molar ratio NAD/G3PDH = 5.9) and a low concentration of ammonium sulfate was donated by Dr. E. Racker. The apoenzyme was isolated by chromatography at about 2° C on a short column (ca. 5 mm) of a mixture of active carbon and Sephadex G-25 dextran gel. The charcoal (Norit A, neutral) was purchased from Fisher Scientific Co. and was purified according to the method of Krinsky et al.¹⁰⁵. The dextran gel (Pharmacia, Uppsala) was washed in water before use. The activity of the enzyme toward G3P was determined by the method of Krinsky et al.¹⁰⁵. The absorbancy ratio: A_{280}/A_{260} was used as evidence of the absence of significant quantities of denatured protein and of impurities of purine and pyrimidine derivatives. This requirement is satisfied when the ratio has a high value. The best preparations had an A_{280}/A_{260} -ratio of about 2. A specific extinction at 280 nm of 0.908 for a 0.1% solution of the protein was used¹¹⁵.

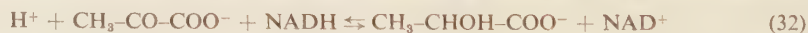
The holoenzyme was prepared by reconstitution from the apoenzyme and excess NAD (6-23 moles NAD/moles enzyme)¹¹⁶. The molecular weight was assumed to be 140000¹¹⁷. The G3P was purchased from Boehringer & Söhne, Mannheim (grade A, assay: 99%) as barium salt of the diethylacetal. The acetal was hydrolyzed and the Ba-ions were exchanged with protons by treatment of a suspension of 25 mg of the substance and 1.9 ml moist Dowex-50 resin (H⁺-form) in 5 ml water for 3 min. with intermittent shaking in a boiling water bath.

The hemimercaptal-enzyme complex (an addition compound of the sulphydryl group to the aldehyde group of the substrate) was formed by addition of about 4000 fold G3P to the apoenzyme at pH 7.7 and room temperature. This forces the equilibrium:



practically quantitatively (99.9%) toward the complex.

After the measurement of the optical rotatory dispersion, about 6 moles of NAD per mole enzyme were added to form the holoenzyme and expel the product (3-phospho-glyceric acid, (3-PGA)). The latter, as well as NADH and excess NAD, was removed by Sephadex chromatography. NADH is easily removed, since the stability constant for its complex with the apoenzyme is far smaller than that of NAD and the binding site is the same^{105, 118}. NADH is a potent inhibitor of the G3P-enzyme formation and must therefore be removed¹¹⁹, e.g. by the reaction:



which is catalyzed by lactic dehydrogenase. The completeness of the removal of products and cofactors was tested by a specific assay for 3-PGA¹²⁰ and by measurement of the absorbancy ratio (A_{280}/A_{260}). The latter must have a value near 2. After the measurement of the optical rotatory dispersion of the holoenzyme, the activity of the enzyme was determined as described above.

The substrates, cofactors, ATP, pyruvate kinase, phosphokinase and lactic dehydrogenase were purchased from Boehringer & Söhne. The water was distilled, deionized and outboiled. All other reagents were Mallinckrodt chemicals of AR-grade.

Instruments: The optical rotatory dispersion (O.R.D.) measurements were made with a Rudolph High Precision spectropolarimeter with photoelectric attachment and rocking analyzer as previously described⁸. The temperature was kept constant within 0.3° C with circulating water. The measurements were made in a 2 dm quartz polarimeter cell with water jacket (Optical

Cell Co., Kensington, Md., U.S.A.). The lamp was a Hanovia xenon-mercury concentrated arc lamp from Engelhart Industries (Newark, N.J., U.S.A.).

All spectrophotometric measurements were made with a Cary-14 recording spectrophotometer (Applied Physics Corp., Monrovia, California). The protein concentration in the polarimeter tube was 0.6–1.8 mg/ml. The pH-measurements were made with a Radiometer pH-meter model TTT1.

Results

The dispersion curves were obtained by subtraction of the appropriate solvent curves from the directly observed data. The adherence of the data to the Drude law of optical rotatory dispersion:

$$-[\alpha]_{\lambda} = K/(\lambda^2 - \lambda_c^2) \quad (33)$$

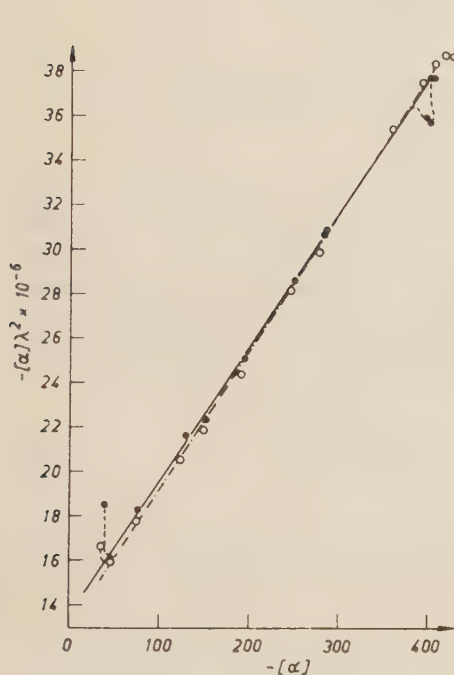


Fig. 30

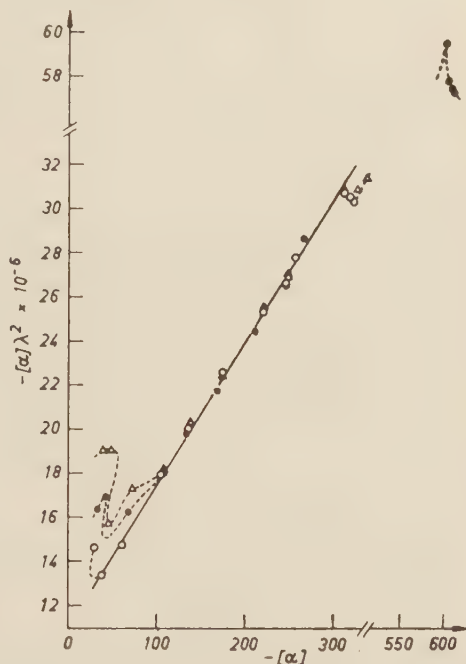


Fig. 31

Figure 30

Optical rotatory dispersion of glyceraldehyde-3-phosphate dehydrogenase apoenzyme in 0.15 *M* KCl, 0.001 *M* EDTA at 23° C plotted according to the one-term Drude equation omitting the points which are significantly influenced by the anomalous rotation near 305 and 650 nm. See text. Symbols: ○, pH 8.4, stippled line; ●, pH 7.7, full line. The lines were calculated by linear regression. Broken lines: Anomalous rotatory dispersion (probably Cotton effects).

Figure 31

Optical rotatory dispersion of the apoenzyme in 0.15 *M* KCl, 0.001 *M* EDTA, 6×10^{-4} *M* α -thioglycerol and various concentrations of NAD at pH 7.70 and 23° C. Plot: As in Fig. 30. Symbols: ○: 0.0 [NAD]/[Enz.], molar ratio; ●: 9.2 m.r.; Δ: 23.0 m.r. Molecular weight of the protein: 140000. The full line in the graph is the regression line for [NAD]/[Enz.] = 0. The broken lines indicate the contour of the anomalous rotatory dispersion.

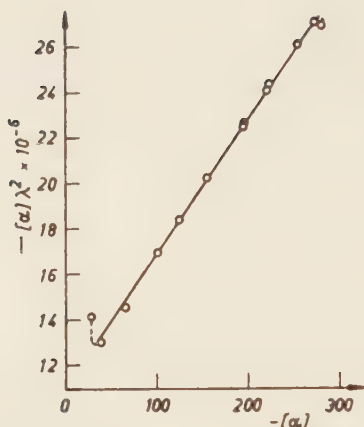


Figure 32

Optical rotatory dispersion of the G3P-hemimercaptalenzyme in 0.15 *M* KCl, 0.001 *M* EDTA, 0.081 *M* G3P at pH 7.7 and 23°.5 C. Line: Regression line.

where $[\alpha]_{\lambda}$ = specific rotation at the wavelength λ (nm), K = constant, λ = wavelength of incident light and λ_c = critical wavelength, was tested graphically in the range 300–700 nm (Figs. 30–32). The data are summarized in Tables 7–9. Table 6 shows the experimental conditions, some criteria of the quality of the preparations and the characteristics of the anomalous rotation effects, tentatively ascribed to Cotton effects^{121, 122}. The O.R.D. parameters and the derived estimates of the helical content are presented in Table 7. The parameter b_0 was determined with the aid of the Moffitt equation^{73, 123–127}:

$$[m'] = (a_0\lambda_0^2/(\lambda^2 - \lambda_0^2)) + (b_0\lambda_0^4/(\lambda^2 - \lambda_0^2)^2) \quad (34)$$

where $[m']$ = mean residue rotation = $[\alpha](M_0/100)(3/(n^2 - 2))$, $[\alpha]$ = specific rotation, M_0 = mean residue weight = mol.wt./total number of AA, AA = amino acid and n = refractive index.

The value of λ_0 was chosen to be 212 nm, because this value gives a good fit of the data to the equation for poly- γ -benzyl-L-glutamate and for many proteins in a variety of solvents⁷³. The dispersion of the refractive index was approximated with that of water¹²⁸. The value of the parameter b_0 was calculated from a Moffitt plot ($[m'](\lambda^2 - \lambda_0^2)$ vs. $1/(\lambda^2 - \lambda_0^2)$). The slope of the regression line is $-b_0\lambda_0^4$. The apparent helical content was evaluated from the equation:

$$\% \text{ helix} = -b_0/630 \quad (35)$$

Discussion

An interpretation in details of optical rotatory dispersion data for proteins is difficult in the present state of our knowledge of the theory¹⁷⁰, but the method provides a very sensitive indicator of the occurrence of conformational changes

Table 6

Protein	Enz. mg/ml	[NAD]/ [Enz.] ^a	pH	Solvent	Temp. °C	Anomal. rot. ^d log A _{max} nm		$\frac{A_{280}}{A_{260}}$	Activity ^b min ⁻¹
						# 1.	# 2.		
0.....	0	—	7.42	$6 \times 10^{-5} M \text{ ZnCl}_2$ $3 \times 10^{-5} M \text{ NAD}$	20.1 ± 0.2	310 ± 5	—	—	—
0.....	0	—	7.5	$0.11 M \text{ KCl}$, $0.04 M$ tris	20.1 ± 0.2	310 ± 5	—	—	—
Apoenzyme.....	0.775	0	8.4	$0.15 M \text{ KCl}$, $0.001 M \text{ EDTA}$	23.5 ± 0.5	310 ± 10	675 ± 30	1.66	$1.8 \times 10^6 \text{ Fe}$
Apoenzyme.....	0.588	0	7.7	$0.001 M \text{ EDTA}$, $0.15 M \text{ KCl}$	23.5 ± 0.6	310 ± 10	675 ± 30	1.85	$1.17 \times 10^6 \text{ Fe}$
Apoenzyme.....	1.87	0	7.70	$0.15 M \text{ KCl}$, $0.001 M \text{ EDTA}$, $6 \times 10^{-4} M \alpha$ -thioglycerol	23.5 ± 0.4	310 ± 10	650 ± 30	1.50	$2.50 \times 10^6 \text{ Fe}$
Holoenzyme (w. NAD).....	1.64	9.2	7.70	$6 \times 10^{-4} M \alpha$ -thioglycerol	23.4 ± 0.5	312 ± 8	630 ± 30	—	$1.43 \times 10^6 \text{ Fe}$
Holoenzyme (w. NAD).....	1.06	22.5	7.70	$6 \times 10^{-4} M \alpha$ -thioglycerol	23.2 ± 0.2	312 ± 8	600 ± 40	—	$0.37 \times 10^6 \text{ Fe}$
Hemimercaptalenzyme ^c	1.70	0	7.7	$0.15 M \text{ KCl}$, $0.001 M \text{ EDTA}$, $0.081 M \text{ G3P}$	23.4 ± 0.1	312 ± 8	640 ± 30	1.29	$0.012 \times 10^6 \text{ Fe}$

^a Molar ratio based on a molecular weight of 140000 of the protein.

^b Racker found: $8.0 \times 10^6 \text{ min}^{-1}$ at 27°C , pH 8.6, assuming a mol. wt. of 130000.

^c $[\bar{E}]/[\bar{ES}] - K_s/[\bar{S}] \simeq K_M/[\bar{S}] - 2.2 \times 10^{-5}/0.0805 - 2.74 \times 10^{-4} \simeq 99.97 \pm \text{ES (hemimercaptal-enzyme)}$. E = apoenzyme; S = G3P; K_s = $[\bar{S}]/[\bar{E}]/[\bar{ES}]$; K_M = Michaelis constant.

^d The anomalous rotatory dispersion is tentatively assumed to be due to Cotton effects. The origin of effect No. 1 is probably a Zn-complex^{121, 122}. The origin of No. 2 may be sulphur, since NAD binds near cystein^{112, 171}.

^e F = forward reaction: $\text{G3P} \rightarrow 3\text{-PGA}$.

Table 7

Protein	pH	[NAD]/[Enz.] Molar ratio	λ_c , nm ^a	$-K^a$ c. deg (nm) ² (g/100 ml) dm $\times 10^{-6}$		r^b	$-b^c_0$ Circ. dg.	Apparent helical content ^c %	No. of obs.
Apoenzyme.....	8.4	0	249.8 $\pm$ 1.7	13.0 $\pm$ 1.6		0.983	154 $\pm$ 6	24 $\pm$ 1	11
Apoenzyme.....	7.7	0	246.2 $\pm$ 1.1	13.6 $\pm$ 1.0		0.989	131 $\pm$ 6	21 $\pm$ 1	11
Apoenzyme + α -thioglycerol,	7.70	0	255.0 $\pm$ 0.2	11.00 $\pm$ 0.17		1.001	158 $\pm$ 6	25 $\pm$ 1	8
Holoenzyme (w. NAD) + α -thioglycerol.....	7.70	9.2	253.8 $\pm$ 0.5	11.1 $\pm$ 0.4		1.004	158 $\pm$ 6	25 $\pm$ 1	9
Holoenzyme (w. NAD) + α -thioglycerol.....	7.70	22.5	252.6 $\pm$ 0.9	11.3 $\pm$ 1.4		0.950	158 $\pm$ 6	25 $\pm$ 1	7
Hemimercapital-enzyme,	7.70	0	242.9 $\pm$ 0.8	11.0 $\pm$ 1.1		0.972	98 $\pm$ 6	16 $\pm$ 1	11

^a λ_c and K were calculated by linear regression. Only the observations, which were considered to be free of significant perturbation due to the anomalous rotatory dispersion effects at 305 and 650 nm, were used.

^b r = correlation coefficient.

^c See text.

Table 8

Comparison	Parameter	Significance of difference
Apoenzyme, pH 8.4 vs. pH 7.7.....	λ_c	+
	K	—
	b_0	—
Apoenzyme with α -thioglycerol vs. apoenz. without this compound; pH 7.7.....	λ_c	+
	K	+
	b_0	+
Holoenzyme, pH 7.7, [NAD] / [Enz.] = 9 vs. 23.....	λ_c	—
	K	—
	b_0	—
Apoenzyme, pH 7.7, w. α -thioglycerol vs. holoenz. w. 9 [NAD] / [Enz.], pH 7.7, w. α -thioglycerol.....	λ_c	+
	K	—
	b_0	+
Apoenzyme, pH 7.7, vs. hemimercaptalenz., pH 7.7..	λ_c	+
	K	+
	b_0	+

Table 9

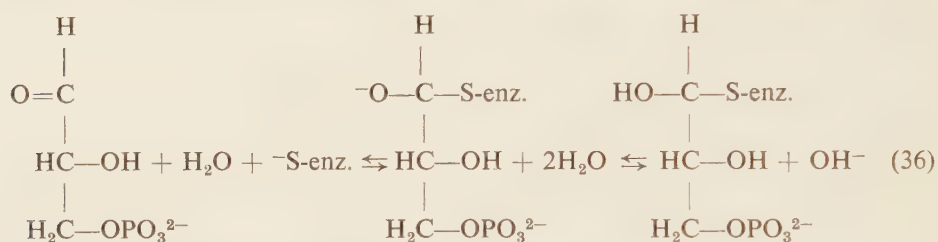
Protein	Range used for calculation of b_0 nm
Apoenzyme at pH 8.4.....	320–500
Apoenzyme at pH 7.7.....	320–500
Apoenzyme + α -thioglycerol.....	325–400
Holoenzyme (w. NAD) + α -thioglycerol, m.r. 9.2.....	325–400
Holoenzyme (w. NAD) + α -thioglycerol, m.r. 22.5.....	325–400
Hemimercaptalenzyme.....	320–520

in general. It is also possible to make a crude classification of the major changes into those representing alteration of the helical content and those involving the solvation of centres of asymmetry from the changes in b_0 (or λ_c) and a_0 (or K)^{129, 130}, respectively. The location of the changes in the molecule, however, must be determined by a different method. In view of the recent findings by Litman et al.¹³², that other peptide bond interactions than helix formation sometimes can contribute to b_0 , it is necessary to consider the estimate of the helical content based on this parameter as a first approximation. The error does not appear to be very common or large.

G3PDH is a protein with a relatively high helical content according to estimates based on the amino acid composition^{129, 130, 133} or on b_0 . It is, therefore, not surprising that even a small pH-change significantly alters the helical content (Table

8 and Fig. 30). Addition of the non-polar solvent, α -thioglycerol, even at a low concentration, changes both the apparent helical content and the solvation of one or more of the centers of asymmetry (Table 8). The question of whether the effect of α -thioglycerol on the O.R.D. parameters is specific or general, is important, but remains to be answered. Binding of the stoichiometric quantity of NAD to the enzyme alters the apparent helical content, but not K . The presence of excess of NAD does not appear to give rise to any further changes in the dispersion curve with the exception of an enhancement of the anomalous dispersion effect at 650 nm (Table 8 and Fig. 31).

The formation of the enzyme-substrate intermediate in the forward reaction, G3P-hemimercaptal-enzyme, changes both the helical content and the symmetry (Table 8 and Fig. 32). The following reaction scheme has been proposed:



The overall stability constant, $K_{\text{stab}} = [\bar{\text{E}}\bar{\text{S}}]/[\bar{\text{E}}] \times [\bar{\text{S}}]$, is small ($4.5 \times 10^{-4} M$). The measurements must therefore be made in the presence of a large excess of substrate which forces the equilibrium in the direction of ES.

Preliminary measurements of the optical rotatory dispersion parameters of the enzyme-substrate intermediate in the reverse reaction, 3-phospho-glyceric acid thiol-ester-enzyme, indicate that the parameters b_0 , K and λ_c are significantly different from those of the apoenzyme (at pH 7.7) and the hemimercaptal-enzyme. Thus, it appears that G3PDH also undergoes conformational changes accompanying its interaction with 1,3-diphospho-glyceric acid and that there exists at least two intermediates in the catalytic reaction, G3P-hemimercaptal-enzyme and 3-PGA-thiolester-enzyme, of individual conformation.

Further statements about the nature of the conformational changes probably require the application of more direct methods of structural analysis than optical rotatory dispersion.

Conclusions

1. The apparent helical content of the glyceraldehyde-3-phosphate dehydrogenase apoenzyme is pH-dependent in the range: pH 7.7–8.4 but the solvation (as represented by K) is not.
2. Stoichiometric binding of the cofactor NAD to the apoenzyme alters the apparent helical content (b_0), but not the solvation as represented by a_0 or K^{73} .

Addition of excess NAD causes no further changes except in the amplitude of the anomalous rotation (at 650 nm).

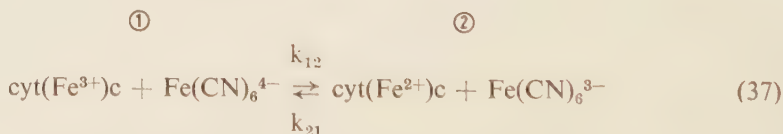
3. Even a low concentration ($6 \times 10^{-4} M$) of the non-polar compound, α -thio-glycerol affects both the helical content (b_0) and the solvation of the apoenzyme (K).

4. Formation of the substrate-G3P-enzyme complex from the apoenzyme changes both the apparent helical content (b_0) and the solvation (K). Anomalous rotation was observed in two wavelength ranges: One at about 305 nm, the other at about 650 nm. The reference curve indicated that the former was due to a Zn-complex (probably a Cotton effect), while the latter might arise from an interaction involving the sulphur atom of cysteine since the sulfhydryl group is part of the active site^{112, 104} and since complexes involving sulphur, (possibly with histidine) can absorb near 650 nm. Histidine has also been implicated in the active site of G3PDH¹.

3.3. CYTOCHROME C

3.3.1. The kinetics of the interaction with the cyanoferrate couple

Since the concept of a catalytic role of conformational changes by no means is restricted to substrate conversion, a natural extension of the investigation was a search for the occurrence of conformational changes in pure electron transfer reactions. The electron transfer in the respiratory chain has a great physiological importance. Therefore, the project was launched with a study of a cytochrome. The best known and most soluble of these is cytochrome c. This catalytic protein was consequently chosen for the preliminary experiments. The results of these confirmed the expectations that the electron transfer in the respiratory chain indeed is accompanied by protein isomerizations. There are also reasons to expect that these are of no less significance to the biological action than those found in the enzymes. This is born out by the work of Greenwood⁶⁷ and by Hess and Czerlinski⁶⁸ as well as by the data presented here. In addition to the structural implications, the method used in this work, a chemical relaxation method, yields rate constants of elementary steps in the electron transfer. Such parameters have not previously been available, because the first application of relaxation techniques by Chance¹³⁵ was made *in vivo*, i.e. on the total respiratory chain with all its ramifications. The cytochroms are, due to the presence of the strong porphyrin chromophore, particularly suitable for studies of elementary steps. Many other biological pigments, however, can now in a similar way be investigated and it may be expected, that much clarification of biochemical reactions will result from such work. The reaction:



has previously been studied by Sutin and Christman¹³⁶, who measured k_{21} with a stopped-flow apparatus. The other rate constant, k_{12} , was not measured by that technique. The expression for the relaxation rate, $1/\tau$ (τ = relaxation time), for the equilibrium above (37), is:

$$1/\tau = k_{21}(K(c_1 + c_0 - 2x) + 2x) \quad (38)$$

where c_1 = initial concentration of $K_4Fe(CN)_6$

c_0 = initial concentration of ferricytochrome c

x = equilibrium concentration of ferrocycytochrome c

x = equilibrium concentration of ferricyanide

$x = (-c_1 + \sqrt{c_1^2 + 4c_1c_0/K})(K/2)$, since $c_0 < c_1$ and $K < 1$,

$$K = k_{12}/k_{21}$$

(39)

Experimental

Materials: The protein was a saltfree, twice crystallized preparation of horse heart ferricytochrome c isolated by the method of Keilin and Hartree¹³⁷. It was purchased from Boehringer & Söhne, Mannheim. The affinity of the preparation for carbon monoxide was tested by the method of Tsou¹³⁸ to ensure, that the material was in the native state. Only an insignificant amount of the gas was found to be bound.

The buffer substances: Potassium phosphate, sodium acetate and tris(hydroxymethyl)amino-methane were purchased as analytical reagents from Merck. Potassium ferricyanide, potassium ferrocyanide, ferrous sulfate, potassium nitrate, p-phenylene diamine, sodium dithionite and dichlorophenolindophenol were also Merck products (pro analysis), while protoporphyrin IX and L-histidine monohydrochloride were purum-reagents from Fluka. All solutions were prepared with redistilled, degassed water.

Instruments: The chemical relaxation experiments were carried out by the temperature-jump method as described by Eigen and DeMaeyer⁸⁶. The initial temperature was 4.°5 C and the final 12° C. The reaction of cytochrome was followed by measurement of the absorbancy changes in the α -band (540 nm) which is characteristic for the reduction of cytochrome c. The desired wavelength was selected by use of an interference filter from Jena Glaswerke (Schott & Gen.). The bandwidth was 14 nm. The changes in absorbancy were recorded on a Textronix oscilloscope and the curve displayed was photographed with a Robot camera.

The pH was measured at 23° C with a Radiometer pH-meter model 22r after calibration with NBS-standard buffers⁸⁵. The spectra were during the spectrophotometric titrations recorded at 12.°9 C, while the assays for cytochrome c were made at 23° C. All spectra were measured with a Beckman DK2A ratio-recording spectrophotometer using thermostatted cuvettes. The potentiometric measurements were carried out with the pH-meter using a Radiometer platinum electrode. The temperature was controlled by a water jacket which surrounded the sample vessel (13.°9 C).

Theory

The expression for the concentration dependence of the relaxation time may be derived as follows: A terminology similar to that in the previous sections (3.1.2. and 3.1.3.) will be used. The instantaneous concentration of a species representative of state ① is: $c_1 = c_1^0 + x_1$ where c_1^0 is a time independent reference concentration and x_1 is the concentration change due to the sudden temperature rise. A similar expression is valid for state ②. The equilibrium concentration corresponding to the elevated temperature is:

$$c_1 = c_1^0 + \bar{x}_1 \text{ and similarly for state ②.}$$

The instantaneous concentrations of the components are therefore:

$$\text{For } [\text{cyt}(\text{Fe}^{3+})\text{c}] = x_1^{\text{cytFe}^{3+}} + c_1^0 \quad (40)$$

$$\text{For } [\text{Fe}(\text{CN})_6^{4-}] = x_1^{\text{FCN}^{4-}} + c_1^0 \quad (41)$$

$$\text{For } [\text{cyt}(\text{Fe}^{2+})\text{c}] = x_2^{\text{cytFe}^{2+}} + c_2^0 \quad (42)$$

$$\text{For } [\text{Fe}(\text{CN})_6^{3-}] = x_2^{\text{FCN}^{3-}} + c_2^0 \quad (43)$$

The approach to the new equilibrium state can be described by the equation:

$$\begin{aligned} -\frac{dc_1}{dt} &= -\frac{dx_1}{dt} = -k_{12}[\text{cyt}(\text{Fe}^{3+})\text{c}][\text{Fe}(\text{CN})_6^{4-}] + k_{21}[\text{cyt}(\text{Fe}^{2+})\text{c}][\text{Fe}(\text{CN})_6^{3-}] \\ &= -k_{12}(x^{\text{cytFe}^{3+}} + \bar{c}^{\text{cytFe}^{3+}} - \bar{x}^{\text{cytFe}^{3+}}) \\ &\quad (x^{\text{FCN}^{4-}} + \bar{c}^{\text{FCN}^{4-}} - \bar{x}^{\text{FCN}^{4-}}) + k_{21}(x^{\text{cytFe}^{2+}} + \bar{c}^{\text{cytFe}^{2+}} - \bar{x}^{\text{cytFe}^{2+}}) \\ &\quad (x^{\text{FCN}^{3-}} + \bar{c}^{\text{FCN}^{3-}} - \bar{x}^{\text{FCN}^{3-}}) \end{aligned} \quad (44)$$

If the usual approximations, which are valid for small perturbations of the equilibrium ($< 1\%$), and the definition of the relaxation time are used, then the rate expression may be simplified to:

$$1/\tau = k_{12}(\bar{c}^{\text{cytFe}^{3+}} + \bar{c}^{\text{FCN}^{4-}}) + k_{21}(\bar{c}^{\text{cytFe}^{2+}} + \bar{c}^{\text{FCN}^{3-}}) \quad (45)$$

Introduction of the initial concentrations and the restriction that $c_0 < c_1$ and $K < 1$ leads to equation (38).

Results

The data from the temperature-jump experiments were plotted with $1/\tau$ as ordinate and the concentration term in equation (38) as abscissa. The line, which goes through the origin of the coordinate system, was calculated by linear regression. Such a plot is shown in Fig. 33. The slope of the line is k_{21} . The indicated error, S_{xy} , is the standard error. The equilibrium constant, K , was determined by spectrophotometric titration (that value was used in Fig. 33) and by measurement of potentials. The other rate constant, k_{12} , was calculated from K and k_{21} (39). The parameters k_{12} and k_{21} can also be evaluated from a plot of $1/\tau$ versus $(c_0 - c_1 - 2x)$ using data in the range where x is independent of $c_1(x = c_0)$. The concentration of c_0 was kept constant in that series. The agreement between the values of K , k_{12} and k_{21} determined by different methods and between the experimental and the literature values of k_{21} and the standard potentials, E^0 , is good. (Table 10).

The temperature dependence of the $1/\tau$ -value, which here equals that of $k_{12}(1/\tau - k_{12}c_1, c_1 = \text{const.}, c_1 > c_0 \text{ and } x)$, was found to be only slight (the energy of activation for reaction ① $\rightarrow$ ② = ca. 2 kcal/equiv.) at pH 7.0 and ionic strength 0.17 M (range 12–25°C). Usually, the value for electron transfer falls in the range: 7–14 kcal/equiv.

Table 10
Kinetic and static parameters characterizing the reaction (37).

Origin of data	$k_{21} \times 10^{-7}$ $\text{sec}^{-1} \times \text{M}^{-1} \times 1$	$k_{12} \times 10^{-4}$ $\text{sec}^{-1} \times \text{M}^{-1} \times 1$	$K \times 10^3$	$-E^\circ_{\text{PCN}}$ mV^a	$-E^\circ_{\text{cyt}}$ mV
Ref. 136, 25°, pH 6.0 phos. buf., $\mu = 0.1$	1.6 $\pm$ 1				
This work, 12°, pH 7 tris ^b , $\mu = 0.17$	1.23 $\pm$ 0.10 ^c				
Spectrophot., 12° . 9			0.91 $\pm$ 0.01 ^c		
Measurement of potentials, 13° . 9			1.33 $\pm$ 0.05 ^c		
From k_{12}/k_{21} , 12°			1.27 $\pm$ 0.28		
This work, 14°, pH 7				443 $\pm$ 2 ^{c,d}	297 $\pm$ 2 ^{c,d}
Ref. 141, 14°, pH 7				461 $\pm$ 2 ^{c,d}	260
This work, 19° . 9				460	
This work, 14° . 0					
Ref. 142, 25°					
From $1/\tau$ vs. $(c_0 + c_1 - 2x)$	1.20 $\pm$ 0.30 ^c	1.56 $\pm$ 0.32 ^c			
From $K \times k_{21}$		1.12 $\pm$ 0.27			

a Standard potential of the potassium ferricyanide/potassium ferrocyanide couple referred to the $\text{H}_2/2\text{H}^+$ half cell.

b Tris (hydroxymethyl) amino methane.

c Standard error.

d Measured with Radiometer pH-meter and Radiometer Pt-electrode. Temperature control with water jacket around sample cell.

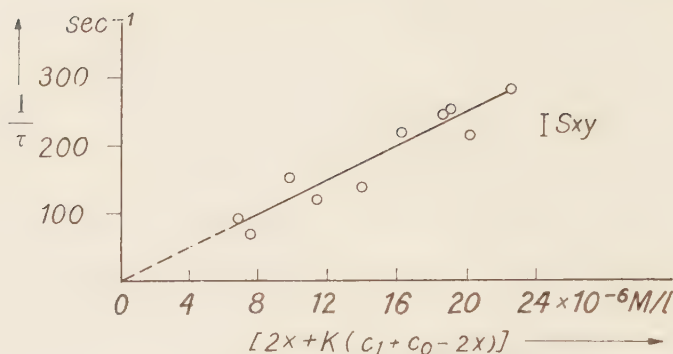
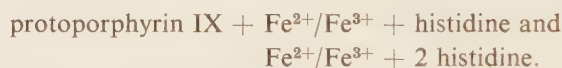


Figure 33

Graph for evaluation of the rate constant of the reaction of ferricytochrome c with potassium ferrocyanide at pH 7.00 and 12° C in 0.14 *M* tris buffer. Ionic strength: 0.17 (tris, KNO₃). Ferricytochrome c (*c*₀): 7.0×10^{-6} – 12.0×10^{-6} moles/l; potassium ferrocyanide (*c*₁): 1.0×10^{-3} – 12.4×10^{-3} moles/l. The line is calculated by linear regression. Slope = $k_{21} \text{ sec}^{-1} \times \text{M}^{-1} \times \text{l}$.

The other symbols: See text. Standard error of the regression: $S_{xy} = \pm 22 \text{ sec}^{-1}$.

An attempt was made to gain some insight in the function of the protein moiety. For this purpose, the following systems, which both lack protein, were tested in the presence of the $\text{Fe}(\text{CN})_6^{4-}/\text{Fe}(\text{CN})_6^{3-}$ couple:



In the latter case, a suspension of a blue complex was soon formed, but the relaxation effect persisted. In both test systems (i.e. with and without protoporphyrin IX), a relaxation effect similar to the one found for complete cytochrome c (but faster) appeared. Thus, the protein is not essential to the appearance of the effect studied. The latter must be associated with the iron atom since histidine cannot participate directly in the electron transfer reaction.

A number of redox couples were tested in an attempt to detect a possible specificity in the interaction with cytochrome c (Table 11). The systems 1–4 appeared to act in accordance with the potential, i.e. the change in free energy.

Table 11

Interaction of ferricytochrome c with a number of redox couples.

System	Reaction	Reduction of $\text{cyt}(\text{Fe}^{3+})\text{c}$	Relaxation effect	Comment
1	$\text{Cyt}(\text{Fe}^{3+})\text{c} + \text{K}_4\text{Fe}(\text{CN})_6$	+	+	See Table 10
2	$\text{Cyt}(\text{Fe}^{3+})\text{c} + \text{dichlorophenol indophenol}$	+	+	$\tau < 10 \mu\text{sec}$
3	$\text{Cyt}(\text{Fe}^{3+})\text{c} + \text{p-phenylenediamine}$	+	+	$\tau < 10 \mu\text{sec}$
4	$\text{Cyt}(\text{Fe}^{3+})\text{c} + \text{Na}_2\text{S}_2\text{O}_4$	+	+	$\tau < 10 \mu\text{sec}$
5	$\text{Cyt}(\text{Fe}^{3+})\text{c} + \text{K}_3\text{Fe}(\text{CN})_6$	—	—	No reaction

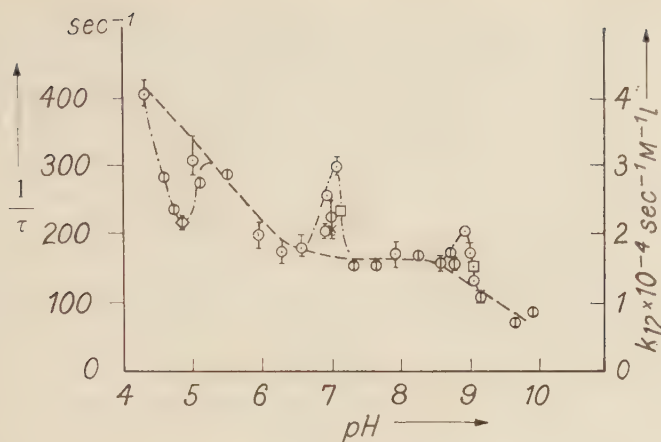


Figure 34

The pH-dependence of the relaxation rate and k_{12} for the cytochrome c-potassium ferricyanide/potassium ferrocyanide system at 12° C. Tris, acetate and phosphate buffers, 0.01–0.15 *M*. Symbols: ○, 0.26 *M* ionic strength; ×, 0.17 *M* ionic strength. Cytochrome c: $2.5 \times 10^{-5} \pm 0.3 \times 10^{-5}$ *M* assuming a molecular weight of 12000. $K_4Fe(CN)_6$: $1.09 \times 10^{-2} \pm 0.03 \times 10^{-2}$ *M*. The estimated error is indicated by a vertical line through the point. Wavelength: 540 ± 5 nm (α -band). Each point represents the average of about 6 observations. In general: One solution for each point. Single observation: ◇. Extrapolated to time of mixing: ◇. --- This curve is parallel to the titration curve. -.- Specific effects.

pH was measured at 23° C instead of at 12°. The pH-values above 7 should therefore be raised about 0.05 pH-unit.

No indication of the importance of specificity was found. Case 5 showed that the relaxation effect only appeared when ferricytochrome c was partially reduced. This confirmed the view that an electron transfer was observed.

Discussion

The pH-dependence of the relaxation rate, $1/\tau$, which within experimental error is proportional to that of k_{12} ($1/\tau = k_{12}c_1$, $c_1 = \text{const.}$), was measured because it might reveal the identity of groups essential to the observed reaction (Fig. 34). The general rise in relaxation rate and in k_{12} toward low pH-values is probably mainly due to changes in the net charge of the molecule which unfolds the protein by electrostatic repulsion. This may make the haem group more accessible to the other redox couple. The narrow peak pH 7.1 may be interpreted as due to catalysis of the electron transfer by two groups, presumably non-haem-bound imidazole sidechains, in a cooperative fashion. In the histidine range (near pH 7), the data can be fitted with an equation of the form:

$$k_{12} \text{ obs} = k_{12}/(1 + [\bar{H}^+]/K_{a1} + K_{a2}/[H^+]) = k_{12}[\bar{\text{cyt}}(Fe^{3+})\bar{H}c]/$$

$$\Sigma[\text{cyt}(\text{Fe}^{3+})\text{c}] = \frac{k_1 K_{a1} K_{a2} + k_2 [\bar{\text{H}}^+] K_{a2} + k_3 [\bar{\text{H}}^+]^2}{K_{a1} K_{a2} + [\bar{\text{H}}^+] K_{a1} + [\bar{\text{H}}^+]^2} \text{ with} \quad (46)$$

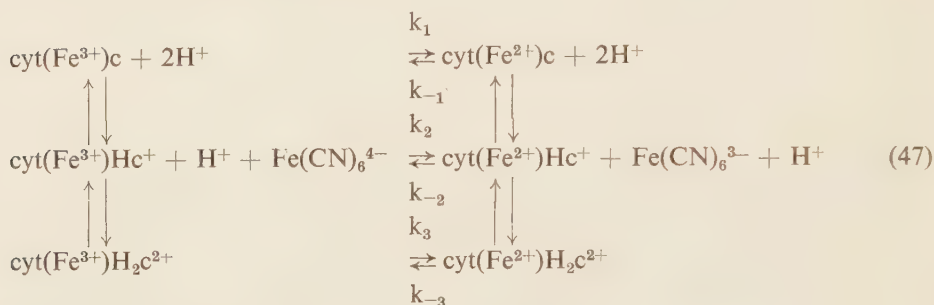
$$k_{12} \text{ obs max} = 8.7 \times 10^4 \text{ l} \times \text{M}^{-1} \times \text{sec}^{-1} \text{ and } pK_{a1} = pK_{a2} = 7.1$$

$$k_2 = 2.61 \times 10^5 - 2k_1 \cong 2.61 \times 10^5 \text{ l} \times \text{M}^{-1} \times \text{sec}^{-1}$$

assuming $K_{a1} = 10^{-7.1}$

and $k_1 = k_3$ for symmetry reasons.

This corresponds to the scheme:



Similarly, the data near pH 9 can be treated:

$$k_{12} \text{ obs} = k_{12}/(1 + [\bar{\text{H}}^+]/K_{a1} + K_{a2}/[\bar{\text{H}}^-]) = k_{12} [\overline{\text{cyt}}(\text{Fe}^{3+})\text{Hc}]/\Sigma[\text{cyt}(\text{Fe}^{3+})\text{c}] \quad (48)$$

$$k_{12} \text{ obs max} = 6.0 \times 10^4 \text{ l} \times \text{M}^{-1} \times \text{sec}^{-1} \text{ and } pK_{a1} = pK_{a2} = 9.0$$

$$k_2 = 1.80 \times 10^3 - 2k_1 \cong 1.80 \times 10^3 \text{ l} \times \text{M}^{-1} \times \text{sec}^{-1}$$

assuming $K_{a1} = 10^{-9.0}$, $\sqrt{K_{a1}K_{a2}} = 10^{-9.0}$

and $k_1 = k_3$ for symmetry reasons.

Scheme (47) is also here valid. The groups responsible are likely to be haeme-bound histidine groups^{139, 140}, since the only α -amino group present carries an acetyl group. However, anomalous ε -amino groups or tyrosyl residues can also have pK' -values in that range.

The rate minimum near pH 4.8 appears to implicate the carboxyl groups either on the protein or on the porphyrin. It is also possible that protonation of $\text{Fe}(\text{CN})_6^{4-}$ plays a role in this range, since its pK' is 4.25 at 25°C. However, the cyanoferrate couple cannot provide the entire explanation because the shape of the curve requires the transfer of 2 protons and only a single proton is bound by that reactant in the pH-range in question. The experimental data suggest a scheme of the type: (47) and the equation:

$$k_{12} \text{ obs} = k_{12}/(1 + [\bar{\text{H}}^+]/K_{a1} + K_{a2}/[\bar{\text{H}}^-]) = k_{12} \Sigma[\text{cyt}(\text{Fe}^{3+})\text{c}] / [\overline{\text{cyt}}(\text{Fe}^{3+})\text{Hc}] \quad (49)$$

$$\text{with } k_{12} = (0.70 \pm 0.10) \times 10^4 \text{ l} \times \text{M}^{-1} \times \text{sec}^{-1}$$

$$\text{and } pK_{a1} = pK_{a2} = 4.8; \sqrt{K_{a1}K_{a2}} = 10^{-4.8}$$

The pH-dependence of k_{12} is probably sooner due to variations in the protein stability than in the resonance energy since the sensitivity of the α -band to pH near neutrality is minor. Moreover, the increase in k_{12} is paralleled by an increase in the rate of autooxidation which is known to be accompanied by protein denaturation and inactivation with respect to coupling with the cytochrome oxidase system. A blue precipitate is formed when a solution of cytochrome c and ferrocyanide is left for 10 hours at pH 4.3–4.5 and room temperature. Iron must have been removed from cytochrome to form a ferrocyanide complex after major protein denaturation. Since protein isomerizations almost invariably are accompanied by changes in the proton equilibria, it is very likely that the pH-dependence, which was found for k_{12} , represents fast proton transfers labilizing the structure prior to the conformational change.

Conclusions

The rate of electron transfer from cytochrome c to the next neighbour in the respiratory chain was relatively slow. The transfer of electrons between the cyanoferrate couple and cytochrome c was also slow when the protein was in its native state, but fast when cytochrome c was denatured. Since there was a good correlation between the pH-dependence of the rate constant for the interaction of ferricytochrome c with ferrocyanide, k_{12} , and the pH-dependence for the stability of the protein, it was concluded that the low polarity of the vicinity of the haem was responsible for the slow electron transfer. Since conformational changes in the cytochrome molecule have been detected, it was possible that the rate of electron transfer was controlled by such protein isomerizations. This was particularly plausible and interesting because cytochrome c represented the rate determining step in the respiratory chain.

The pH-dependence of k_{12} generally followed the protolytic titration curve (and E^0), but specific effects of considerable magnitude were found near (within $\pm 1/4$ pH-unit) pH 7.1, 9.0 (rate increase) and 4.8 (rate decline). The steepness and symmetry of these extrema indicated that the origin was an interaction between pairs of ionizing groups of almost identical pK' . It was probable that these were 2 haem-bound imidazole sidechains, 2 non-haem-bound histidine residues and 2 carboxyl groups.

Chapter 4

The stage of the problem and general discussion

Until a few years ago, the evidence supporting the concept of the occurrence of conformational changes accompanying enzyme catalyzed reactions was rather sporadic, because only very few biochemists considered this phenomenon as an important factor in biological catalysis. By coincidence, pieces of evidence were found in a variety of enzyme systems, but in each case the information was insufficient for a definite conclusion.

Over a period of about six years, the work has mainly been devoted to one system, the interaction of α -chymotrypsin with esters of low molecular weight in order to settle the question of the possible existence and significance of the conformational changes in enzymes definitively. The groups of Hess, Bender, Gutfreund, Bernhard and Lumry have been particularly active in the investigation of this problem.

It was assumed as a working hypothesis, that the catalytic process was accompanied by specific configurational changes in the chymotrypsin molecule and that these changes had kinetic and energetic significance. This hypothesis was tested with the experimental evidence available.

a. The first piece of evidence was negative. The spectrophotometric titrations did not provide any direct evidence of conformational changes during the formation of DIP-CT, but they showed that both α -chymotrypsin and diisopropyl-phosphoryl-chymotrypsin contained two phenol groups with normal and two which had abnormally high pK' -values (above 12). Hence, the conformational change must have occurred in a part of the molecule which was independent of, and presumably far from, the tyrosine residues. An alternative was, that tyrosine only intermittently was involved in formation of the acyl-enzyme intermediate. The observations also excluded the possibility that the phenol sidechains were responsible for, or contributed to, the difference spectrum: α -chymotrypsin versus DIP-chymotrypsin¹⁰. Direct phosphate analysis showed that the diisopropyl-phosphoryl group on the active serine residue was not hydrolyzed during the experiment. This may seem surprising judging from the stability of model compounds, but the result can perhaps be explained by the low polarity of the environment of the active site of chymotrypsin and by steric hindrance. Besides, it had been shown that the reaction responsible for the appearance of the difference

spectrum at 290 nm preceded the actual acylation process, and that conversion of the active serine into dehydroalanine by a β -elimination reaction was independent of the conformational change and associated phenomena, such as the 290 nm difference spectrum or changes in the a_0 -parameter of the optical rotatory dispersion⁶⁰.

b. The optical rotatory dispersion data for α -chymotrypsin, DIP-chymotrypsin, monoacetyl-chymotrypsin and deacylated monoacetyl-chymotrypsin was a piece of evidence which indicated that a changes in the three-dimensional structure of the enzyme molecule had taken place. The sensitivity of this method for detection of conformational changes has abundantly been proven by Djerassi and coworkers¹⁴³. A detailed interpretation of the dispersion parameters of a macromolecule is very difficult, partly because a fully satisfactory theory of optical rotation, especially of large molecules, yet has to be developed, but the O.R.D.-method is useful for comparison between closely related molecules. Basic experimental work on the optical properties of polymers also has been lacking, although it now is emerging. Examples of this is Tanford's study of the effect of solvent polarity on a_0 and b_0 ¹³¹, Imahori, Doty, Blout and Wada's¹⁴⁴⁻¹⁴⁸ work on the contribution of β -structure and Fasman and Goodman's systematic investigation of the optical rotation of synthetic polymers.

The data for all the four enzymes: α -chymotrypsin, DIP-chymotrypsin, monoacetyl-chymotrypsin and deacylated monoacetyl-chymotrypsin accurately fit both the one-term Drude equation and the two-term Moffitt-Yang equation. In the opinion of the author, this indicates that the effect, presumably a resonance loop from a Cotton effect responsible for the optical rotation, is located at a much higher frequency than the range in which the measurements were made. Therefore, the Drude and Moffitt-Yang equations may be considered as Taylor approximations of the true O.R.D.-curve. One or more peptide linkages are probable locations for the electronic transition at which the interaction with the light takes place. The work of Litman and Schellman¹³² on the lactam of 2,4-diaminobutyric acid supports this hypothesis. In non-polar solvents this compound develops a $n-\pi^*$ Cotton effect at 231 nm. Since the present measurements are made from 300 nm to 700, this Cotton effect should be so far from the range of observation that non-linear effects beyond the experimental error are avoided.

The changes in b_0 and a_0 suggest that the acylation of the active site of α -chymotrypsin is accompanied by minor changes in helical content, but major changes in "solvation". The latter are probably due to structural changes other than those involving helical regions. This conclusion is supported by the observation by Tanford of the influence of solvent polarity upon the dispersion parameters of N-acetyl-glutamic acid¹³¹. Tanford found that the solvent polarity greatly affected a_0 , but did not change b_0 appreciably. A simple interpretation of the changes in the chymotrypsin conformation is that previously buried sidechains become exposed to aqueous solvent during the isomerization. This agrees with Wootton's conclusion about the origin of the difference spectrum at 290 nm:

DIP-CT versus CT. Wootton attributed this phenomenon to solvent perturbation of an indolyl chromophore⁴⁰. The perturbing agent is hydrophobic in nature and is therefore likely to be one of the amino acid sidechains.

The pH-dependence of the specific rotation is a source of much structural information e.g. about conformational changes. Free α -chymotrypsin exhibits a considerable pH-dependence of the rotation, whereas monoacetyl-chymotrypsin and DIP-chymotrypsin hardly has any. Hence, the enzymes display individual properties.

c. The thermally induced transitions of α -chymotrypsin, DIP-chymotrypsin and monoacetyl-chymotrypsin are reversible at pH 2.0 and low ionic strength. The thermodynamic parameters, the standard enthalpy and entropy of transition and the transition temperature allow a distinction between the three molecules. This supports the tested hypothesis (conformational changes accompanying catalysis).

The changes in specific rotation greatly vary from enzyme to enzyme. It is also apparent, that the thermal phase transition under the present conditions (pH 2, low ionic strength) only partially unfolds the molecules. Further evidence of this was collected by Mercouroff, Hess and Labouesse. They found that the absorbancy at 290 nm of solutions of α -chymotrypsin, DIP-chymotrypsin and O-cinnamoyl- α -chymotrypsin (Cin-CT) increased two-fold after limited proteolysis by pepsin at temperatures causing appreciable reversible denaturation. In the same experiments, it was clear that cinnamoyl-chymotrypsin had a transition temperature which was about five degrees higher than that of DIP-chymotrypsin. This indicates the importance of the structure of the acyl group to the thermal stability of the modified enzyme. Addition of urea also produces changes much larger than those resulting from thermal denaturation. A consequence of the incomplete unfolding process is that the free enzyme and the acyl-chymotrypsins are not quite identical entities in the denatured state. It is therefore not possible to calculate the thermodynamic parameters (energy of activation) characterizing the acylation process from the thermal transition data.

The differences between the results from the various lots of chymotrypsin from the manufacturer were found to be significant¹⁴⁹, but this did not invalidate the conclusions as these are based on effects which are about five times greater than the differences due to the genetic inhomogeneity and slight variations in the isolation procedure. Although it is known that peptides can bind to proteins, this is not likely to take place at the active site of chymotrypsin since the principal subjects of suspicion of the phenomenon, the peptides released by the activation of the zymogen, seryl-arginine and threonyl-asparagine, lack substrate specificity and they are easily removed by dialysis. The thermodynamic parameters of reversible, thermal denaturation were obtained after such a purification by dialysis. Furthermore, the fact, that acylenzymes of α -chymotrypsin were more easily disencumbered of peptide impurities than the free enzyme, was utilized. Mono-acetyl-chymotrypsin was dialyzed and then deacylated before the specific rotation was measured. Subsequently, diisopropyl-fluorophosphate in propanol-2

was added to one aliquot of the solution and the same volume of propanol-2 to another. The usual difference spectrum at 290 nm appeared. Hence, this spectrum cannot be due to peptide impurities, but presumably to conformational changes in the enzyme.

The difference spectrum persists in eight molar urea solution. Yet, under these conditions the molecules should be sufficiently unfolded to release the adsorbed peptides during dialysis. Moreover, Wootton showed that twelve times recrystallized, chromatographically pure chymotrypsinogen, which did not contain any peptide impurities, on activation and subsequent reaction with diisopropyl-fluorophosphate produced the characteristic difference spectrum when its spectrum was measured against that of α -chymotrypsin¹⁵⁰.

e. Finally, Moon, Hess and Sturtevant found that the formation of DIP-chymotrypsin (and monoacetyl-chymotrypsin) exhibited pure bimolecular kinetics²⁶. This is incompatible with the hypothesis of peptide inhibition. The data on specific proflavine inhibition of chymotrypsin is also in disagreement with this hypothesis.

In spite of the large amount of evidence in favour of the occurrence and catalytic significance of conformational changes during chymotryptic action, it does not at the moment seem possible completely to exclude a contribution of an inductive effect to the catalytic process or to the spectral perturbation. If an inductive effect is operating, then it must do so through space rather than through covalent bonds. This has already been discussed by Wootton. Since orbital overlap, e.g. between the electrons of an indolyl group and the C = O or P = O bond attached to the serine hydroxyl group, requires a very short distance between the participants, the acyl group must be rigidly clamped over the aromatic nucleus. This situation is unlikely, but possible. A final conclusion in this question must be deferred, until detailed x-ray crystallographic maps of α -chymotrypsin and DIP-chymotrypsin (or a similar compound) are available. Both the tryptophyl residue and the DIP-group should produce deflections which could be definitively identified even at a relatively low resolution. Such studies are now nearly completed.

Most of the work and the discussion in this thesis has been devoted to chymotrypsin and its intermediates in the catalytic process. However, much evidence corroborating the general conclusion, that reversible conformational changes of profound significance to the catalytic phenomenon are accompanying the substrate conversion, is now available, also for other enzymes such as glyceraldehyde-3-phosphate dehydrogenase, lysozyme and ribonuclease. Also other catalytic proteins, such as cytochrome c and haemoglobin, are known to display very similar effects, conformational changes which result in changes in the optical rotatory dispersion.

The application of chemical relaxation techniques has greatly aided the confirmation of the early static experiments and the kinetic studies using steady-state conditions. The temperature-jump technique has made it possible to evaluate

the rate constants and other parameters of elementary steps in the early phases of the interaction of enzymes with substrates or competitive inhibitors. The kinetic data from the highly sensitive chemical relaxation methods clearly fit mechanisms which include isomerizations of the enzyme-substrate complex, but are incompatible with schemes based on simple binding of substrate to enzyme directly followed by substrate conversion. It is the nature of kinetic methods, that they do not permit a proof of a mechanism, but it is unlikely that kinetic schemes based on data from the sensitive and selective relaxation methods are not correct, since the number of components in the system is quite limited. Therefore, the number of pathways leading from the known reactants over intermediates of known structure to products of known properties is also restricted. Although the x-ray crystallographers so far have been very cautious in their judgement about the question of the occurrence of conformational changes in enzymes accompanying catalysis, they are now prepared to commit themselves to a confirmation of the hypothesis in the case of lysozyme and chymotrypsin. The changes in the deflections i.e. positions of the atoms following substrate binding are definite, though not very far from the limit of resolution. Lysozyme presents a favourable case. Thus, Phillips⁴⁹ has succeeded in observing several stages in the catalytic reaction at a resolution of 2 Å. This is sufficient to identify the sidechains which participate in every step in the catalytic process. Unfortunately, the kinetic studies, which should complement the static investigations, are still in an early stage.

Detailed x-ray crystallographic studies of myoglobin and hemoglobin reveal, that the sidechain-helix (more precisely: sidechain-peptide bond) interactions as often occur between different parts of chains or different parts of the same chain as between segments located in the same section of a chain. Therefore, protein structure and the associated chemical properties must always be considered in terms of the total three-dimensional configuration,—a view which long has been held by many workers in the field. The methods for elucidation of molecular structures have reached a stage of high excellence, especially in the solid state and for crystals wetted with a saturated solution of the substance.

The kinetic techniques, however, which should furnish complementary information about the dynamic properties of the reaction system have lacked behind. Until recently, most kinetic work on catalytic phenomena was performed under steady state conditions or by the stopped-flow method (see p. 19). The former have the advantage, that they are relatively easy to achieve and the progress of the reaction can often be followed with conventional equipment. However, the steady state excludes the observation of important intermediates and the kinetic parameters obtained are complex. Therefore, it is only in certain limiting cases and for simple reactions of minor interest possible to evaluate rate constants and activation parameters for the elementary steps. The number of determinations of complex constants, such as Michaelis' constant and the maximal velocity for various substrates, is very large, and useful information

about important aspects of enzyme catalysis, such as the specificity, has been derived. However, there is no substitute for a knowledge of the parameters of every step in the reaction, when the detailed mechanism is to be elucidated. Therefore, the advent of the development of the chemical relaxation methods, which provide the desired information, was very important. The original techniques were based on transient or periodical perturbation of one or more chemical equilibria under conditions, where components representing each stage in the reaction were present in detectable concentrations. While the steady-state approach is based upon the choice of conditions under which intermediates artificially are suppressed, the original relaxation methods for systems with poised equilibria required a significant coupling between the reaction steps, i.e. the intermediates should be present in concentrations high enough to reveal their existence by demanding due consideration in the kinetic equations. Many enzyme catalyzed reactions have poised equilibria and their kinetic investigation should be straight-forward, although possibly cumbersome. However, there are also many enzyme systems, notably those of hydrolases related to chymotrypsin, which have strongly displaced equilibria. Their reaction velocities for specific substrates under the conditions of maximal activity are too high for measurement by any known technique other than chemical relaxation, but the substrates are converted into products, before it is technically possible to make a relaxation measurement with the original equipment. At present, only a flow system can permit the establishment of an equilibrium with measurable concentrations of reactants and intermediates. Therefore, a combined flow and temperature-jump (T-jump) apparatus was developed (see the appendix). The new instrument, in which a steady-state, instead of an equilibrium in a solution at rest, is perturbed by a sudden temperature rise of about 10 degrees, permitted the measurement of the relaxation times and the rate constants for the elementary steps of a model system. Preliminary studies of the interaction of chymotrypsin with specific substrates using this apparatus have already yielded promising results. Therefore, it appears that this approach can lead to much progress in the studies of the kinetics of a class of chemical reactions, which is important to the understanding of enzyme catalysis. It is, however, not limited to biological reactions, but should be applicable to most branches of solution chemistry. The most important application of the flow-T-jump apparatus to the study of conformational changes in biological macromolecules is likely to be the determination of the number of isomerizations which occur, their location in the kinetic scheme and their rate constants.

Chapter 5

General conclusion and some perspectives

The mechanism of enzyme catalysis has intrigued and challenged generations of biochemists and many others who could appreciate its importance and intricacy. The advance in our understanding of the phenomena in nature, has to a large extent been determined by the rate of development of new techniques. The methods for measurement of reaction rates were rarely more effective than the problems, as the scientists saw them, demanded. Therefore, the elucidation of the detailed structure of enzymes and other proteins, which is the prerequisite for the understanding of any reaction mechanism, became a late start. The improved structural methods recently convinced the workers in the field of enzyme catalysis, that they previously had overlooked important steps and intermediates in the catalytic process and that the otherwise valuable classical steady-state and stopped-flow methods were incapable of yielding the necessary information about elementary steps. The physicists and engineers were then mustered and they produced the chemical relaxation techniques which can provide the desired information. In fact, some of it is already available and more is rapidly forthcoming.

The conformational changes in enzymes accompanying their catalysis are examples of such long overlooked, but essential, steps in the catalytic mechanism. Some foresighted workers early had the feeling that such isomerizations probably occurred, but they were unable to support their contention with conclusive evidence. It is the considered opinion of a person, who keenly has pursued these problems for over seven years, that the first convincing evidence of the occurrence of conformational changes in enzymes accompanying catalysis inadvertently appeared when the difference spectrum between α -chymotrypsin and diisopropylphosphoryl-chymotrypsin was discovered. In spite of many efforts to find other explanations, it was not possible to marshal any other cause of the observed spectral changes than a conformational change in the enzyme accompanying the acylation of the active site with the substrate. Later, direct observation has confirmed the correctness of this conclusion.

This opened a new field of investigation which soon became very popular and a large number of more or less correlated pieces of evidence of the occurrence of conformational changes in a great variety of enzyme systems appeared. The

In all cases, the conformational changes precede the catalytic steps. The latter are now under investigation with the aid of the flow-temperature-jump technique.

The general scheme fits as different catalytic proteins as chymotrypsin, trypsin, glyceraldehyde-3-phosphate dehydrogenase, cytochrome c and haemoglobin and no clear exceptions are known.

The significance of the conformational changes to the catalytic process is, at present, only poorly known, but there is sufficient information for the proposal of hypotheses. Thus, the conformational changes may:

1. serve as means of specificity by failing to respond to the binding of non-specific substrates,
2. channel the reaction into paths with low activation barriers, or
3. may be incidental and trivial.

The last suggestion may perhaps be rationalized by considering the strength required by the protein structure to ensure complete rigidity. The addition to a globular enzyme of almost any non-polar compound, which is small or specific enough to be admitted to the internal of the protein molecule, causes a detectable shrinkage. This is understandable in terms of the decrease in surface energy which is a result of a reduced contact between hydrophobic surfaces and water. The possibility, that the conformational changes are trivial is, however, not likely, since the evolutionary pressures tend to eliminate non-essential effects.

It has been suggested by others and supported by the author, that the great volume of data and the various hypotheses concerning conformational changes in enzymes accompanying their biological action, catalysis, now are about to merge into one general scheme, which has been described above. If this is correct, then a noteworthy milestone in the elucidation of the relationship between the chemical structure and the biological function has been reached.

Chapter 6

Appendix

An apparatus for measurement of very high reaction velocities in unbalanced systems

The reaction velocities found in enzyme catalysis are under optimal conditions in many cases too fast for measurement, even by flow or stopped-flow techniques. Since many enzymes work near the optimal conditions in nature, it was necessary to develop new methods for rate studies to elucidate the reaction mechanism. The chemical relaxation techniques permit measurement of the velocities which come in question, but so far these apparatuses have required the existence of a nearly poised equilibrium, before they could be applied^{95, 96}. Unfortunately, many enzymes operate on systems which are so far displaced from the balanced position, that a transient perturbation fails to elicit a detectable concentration change. Therefore, it was necessary to develop a new method. This was based on perturbation of a steady-state instead of on displacement of a poised equilibrium. The steady-state was maintained for a time (up to 30 sec.) sufficient for application of a transient perturbation in the usual way. This was realized by combination of a flow- and a temperature-jump apparatus^{151, 152}. The time dependence of the approach to the new steady-state was detected by use of the optical density and followed on an oscilloscope. A simple evaluation of the data was only possible, when the steady-state was close to the true equilibrium. Besides, the perturbation must be small enough to permit linearization of the rate equations, i.e. neglectation of terms of higher order than one. Both of these conditions were fulfilled for the test system, which was the electron transfer between p-benzoquinone and the double anion of hydroquinone. It is probable, that the method often is applicable.

The new method may perhaps also be used for the study of fast, simple, oscillating systems. Such phenomena have been theoretically predicted, and they have often been observed in complicated, biological reactions¹⁵³⁻¹⁵⁵. Unfortunately, so far no simple example of this is known.

Since the main application of the flow-temperature-jump technique was intended to be biological problems, the following requirements were given special attention: Flexible operation, minimal consumption of liquid and mild treatment of the reactants. The instrument has proven its usefulness in the following modes of operation: Flow, stopped-flow and temperature-jump experiments and any combination of two of these functions. The smallest volume of reactant solutions for a complete, kinetic experiment is about one milliliter. However, with a slight modification of the construction, it should be possible to perform temperature-jump experiments on a volume as small as 20 microliter.

Description of the instrument

The apparatus consists of a flow system for liquids, a high-voltage discharge circuit and an optical bench (Fig. 35). At first, the individual units will be described since they can operate mutually independently. Then, the device for executing the desired time program will be explained.

The flow system (Fig. 35, C and 35a). The construction of this part was based on the experience of previous workers in the field¹⁵⁶⁻¹⁵⁸ and on the author's own hydrodynamic experiments.

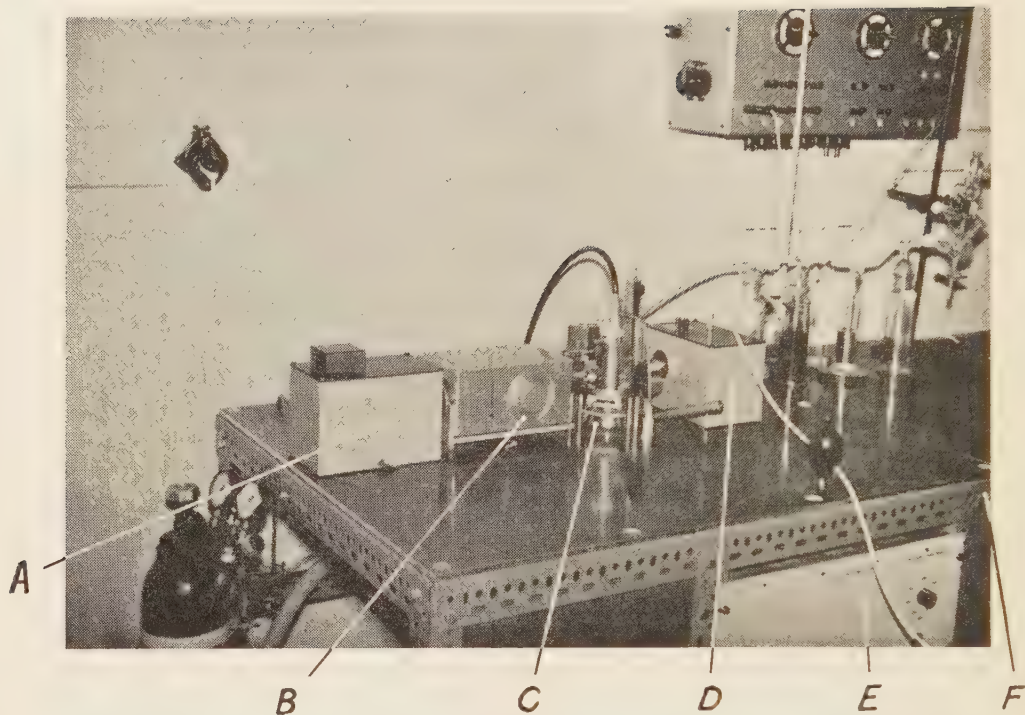
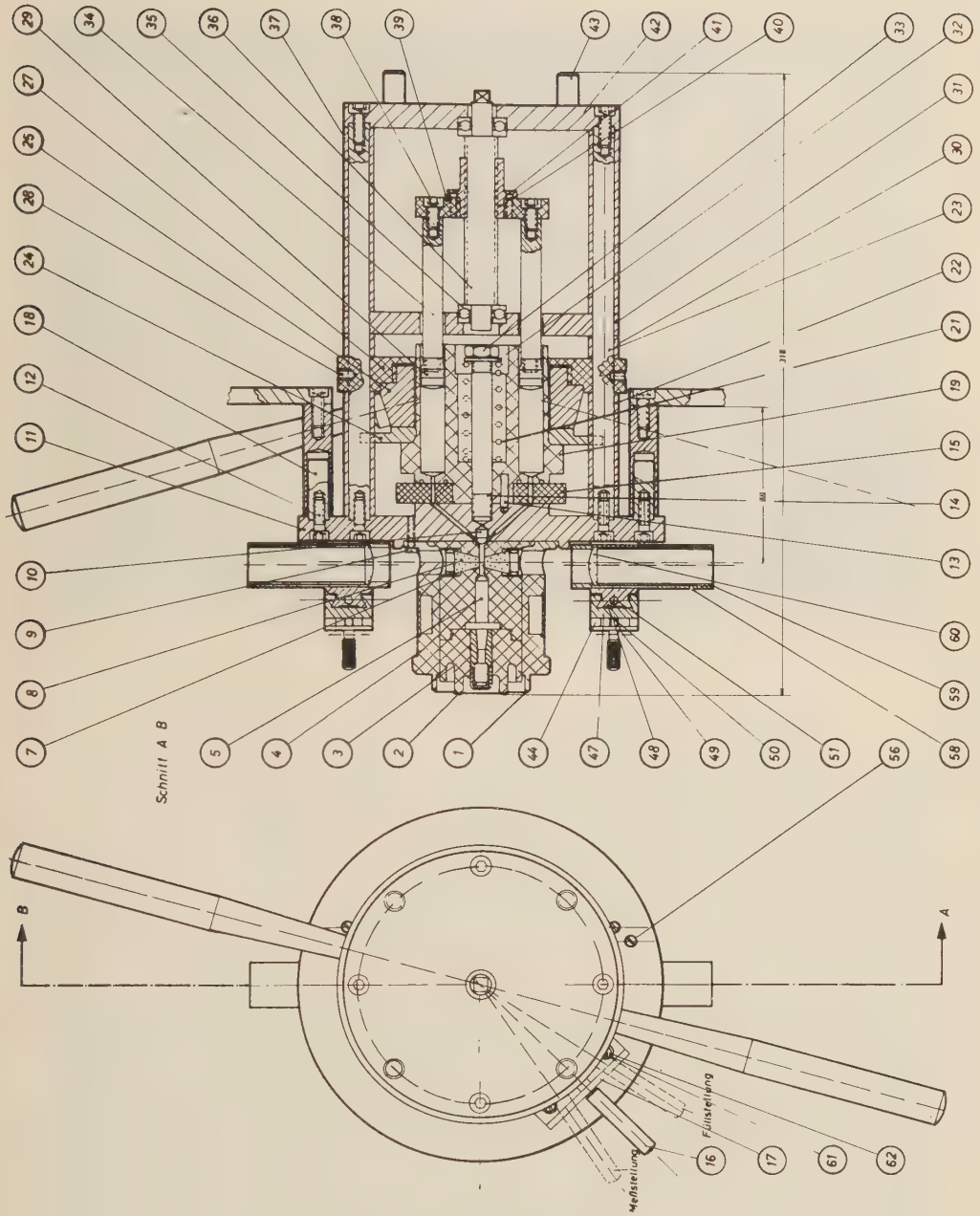


Figure 35

Photography of the flow-temperature-jump apparatus. A: Lamp house. B: Monochromator. C: Flow unit. D: Photomultiplier. E: Synchronization unit. F: High-voltage generator.

In the present construction (Fig. 36a), the two reactant solutions are kept separated until they reach the mixing chamber, but after a small modification of the design, it is possible to mix up to eight different solutions shortly before the observation tube is reached. The temperature of the reactants is controlled in two separated storage containers (total volume 50 ml) with water jacket. This can be done under air-tight cover, protective gas or vacuum. Channels and plastic hoses (1 mm bore) lead the solutions to eight syringes which are drilled in a plexiglass cylinder. The axes of the syringes are parallel to that of the plastic cylinder and are arranged in a symmetrical fashion. They all have the same distance to the main axis of the apparatus. The coaxial design was chosen because it offers maximal mechanical and electronic stability since small vibrations are cancelled. This is important because of the mechanical forces, which come into play when the pistons expel the solution, are strong. The high sensitivity of the detecting system also makes the concentric design mandatory. The rarity of the biological materials require that the dimensions (including the length of the optical light path) are kept small (see Fig. 36a, 37, 38). The signal/noise ratio is therefore critical. A primary source of noise and photomultiplier perturbation is the stray field accompanying the strong surge of current which is needed to heat the reaction mixture and elicit the chemical relaxation. This stray field is minimized, i.e. partially cancelled, when coaxial symmetry is preserved throughout.

The syringes are filled by vacuum from the retreating plungers at low motor speed. When the surface tension of the solutions is low, e.g. in the case of protein solutions, the filling procedure is interrupted about every three seconds to avoid bubble formation by elimination of the pressure difference. The total volume of the syringes is 40 ml, i.e. 5 ml per syringe. Every syringe has a deadspace of about 0.1 ml which is difficult to empty completely. However, the test of Beer's law for a dye was successful and the known rate constant for the interaction of ascorbic acid with dichlorophenolindophenol could be reproduced. Therefore, the dilution error due to the



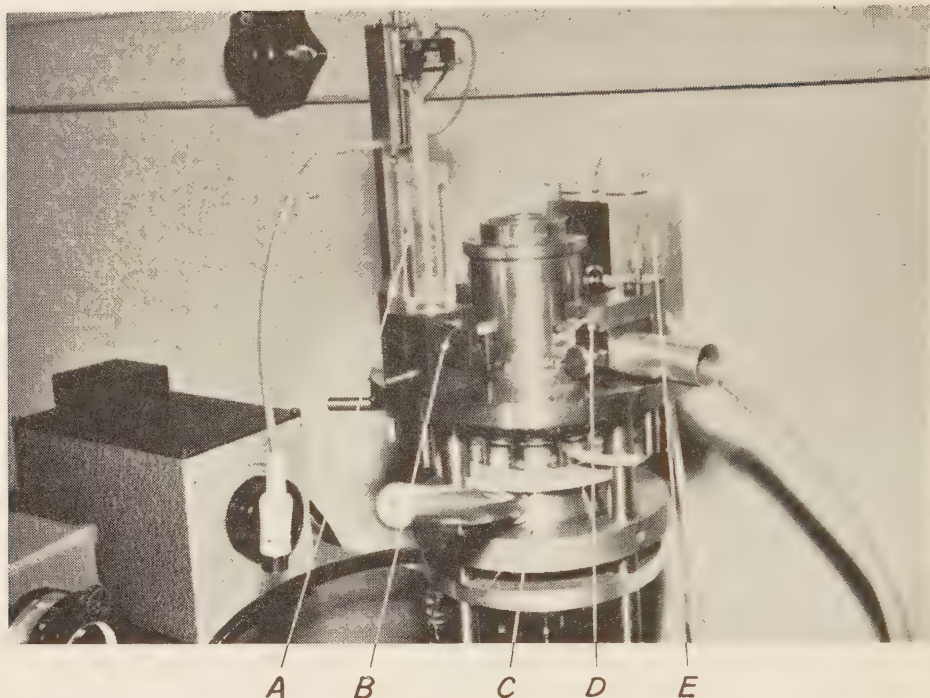


Fig. 36b

Figure 36

a. The flow unit. Cross section through the main axis. 5: High-voltage electrode; 7: Observation chamber; 8: Quartz window; 10: Mixing chamber; 14: Earthed electrode; 15: Cylinder with filling and flow channels; 16: Handled to change position of channel cylinder from filling to flow position; 34: Plunger with rubber head; 36: Driving shaft for motor; 44: Adjustment levers for optics; 60: Quartz lens.

b. Photography. A: Stop syringe; B: Outlet valve; C: Reactant syringe; D: Lens system with levers; E: Reservoirs for reactants.

deadspace is insignificant. The deadspace in the channels is about 0.2 ml. The entry to and the exit from the syringes is a system of channels in a short cylinder which is placed in a coaxial fashion on the syringe block. The entry channels from the reservoirs are eight cylindrical 1 mm passages which each have a 90 degree bend to the side. After the completion of the filling procedure, the channel cylinder is turned $\pi/8$ radians about its axis. This brings the eight straight exit channels in connection with the respective syringes and on the other side, with eight jets (0.40 mm bore). The jets are cylindrical. Their projection on the plane perpendicular to the axis form an angle of 10 degrees to the tangent of the circular perimeter of the mixing chamber at the point where the jet intersects this circle (Fig. 37). The mixing chamber is an annular cavity (passage about 0.5 mm in diameter) between the semispherical surface of the lower electrode and the plastic housing (Macrolon, Bayer or Hostalen, Höchst). The eight jets form parallel spirals and mix about the electrode. The small distance between the jets and the rapid transversal diffusion (i.e. broadening of the spirals perpendicular to the main direction of the flow) enhances effective mixing and creation of desirable turbulence.

A replaceable plastic cylinder with an axial passage (1 or 2 mm in diameter) or with a "Z"-shaped passage (10 mm light path, 1 mm bore) is placed over the lower electrode (the one with the jets at the root). An observation chamber with the same bore as that of the flow tube is

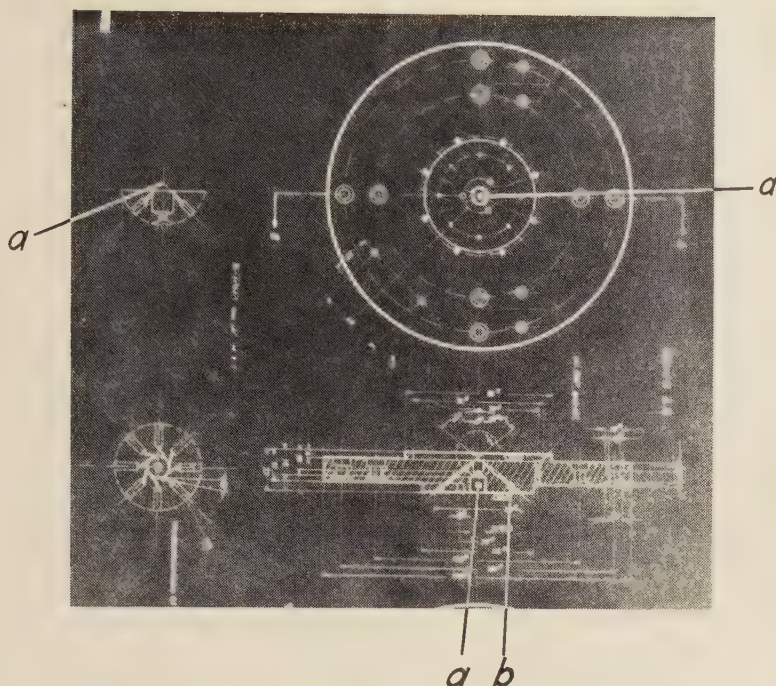


Figure 37

The plate with jets and the lower electrode. Cross sections through the symmetry axis and perpendicular upon this axis. a: Mixing chamber; b: Jet for reactant.

placed at a distance of 12 mm from the mixing chamber (Fig. 38). At the intersection between the two cylinders (for flow and observation, respectively) two conical windows of high grade quartz (12 mm long; diameter of the optical surfaces: 5.6 and 12 mm) are screwed in. A second electrode (cylindrical with semispherical head) is located 4 mm from the observation line in the direction of the flow. The solutions flows around the electrode and through a 2 mm wide exit channel on the side to a stop-syringe (device for fast stop of the flow, Fig. 36b, A), a collection flask or the drain.

The crosssectional area of the flow tube is kept constant from the jets to the outlet port to avoid an unnecessary pressure drop between the electrodes. This considerably reduces the tendency for cavitation and electrical breakdown. The direction of the flow (from below and upwards) facilitates the removal of air bubbles through the reservoirs, if such a complication despite the precautions should arise. During the flow, all parts are tightly pressed together with a tension cone which can be operated by two handles. This precaution prevents the entry of air and loss of material.

A three-way stopcock (of polyvinylchloride or stainless steel) controls the in- and outlets to the stopsyringe (Medical Vitrex, 5 ml) and the bypass to the drain. The stop of the piston is a microswitch (Marquart) which can be accurately adjusted to a run of any length between 0 and 40 mm.

The high-voltage-discharge-circuit. The discharge circuit resembles the one which has been used in apparatuses for non-flowing systems, but the usual Leider condenser was replaced by a coaxial cable. The cable condenser has the advantage that it permits the storage of a high energy and yet gives a steep pulse front due to the low impedance (time constant: $R \times C$, 50 Ω impedance and 100 pF/m). Storage of a high energy is convenient because pulses of a desired duration and energy can be released with the aid of a secondary spark gap without

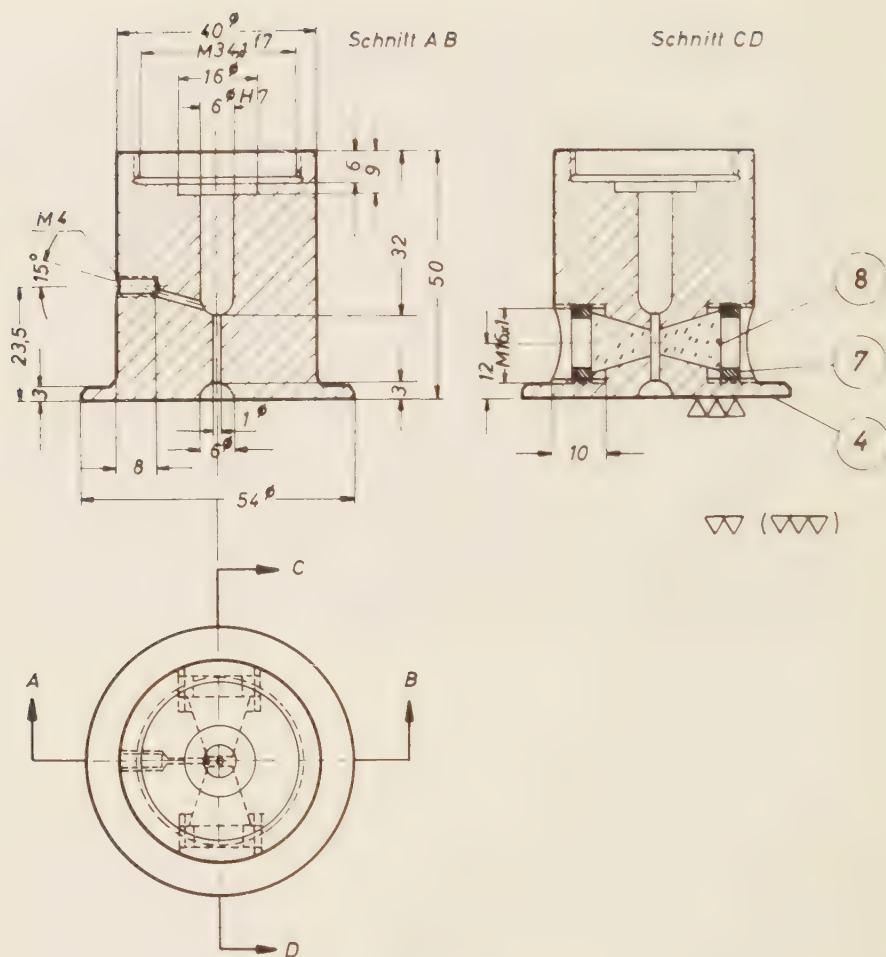


Figure 38
Observation cell. 8: Quartz window.

a recharge of the cable after each experiment. Therefore, it will be possible to apply repetitive discharge to a steady-state, store the relaxation effects for each perturbation and increase the signal/noise ratio by averaging the results. The steep pulse front (an almost rectangular temperature-jump) is advantageous, because it permits penetration deeper into the fast velocity range where many interesting reactions are taken place, and because the data evaluation is facilitated when a correction for the finite time of completion of the perturbation becomes unnecessary. The cancelling effect of the coaxial design on the stray field has already been mentioned. The cable consists of a copper core (3 mm diameter) surrounded by a polythene layer which is covered by a grounded copper screen.

A high-voltage generator (Spellman High Voltage Co., model lab. 90, New York) can charge the cable to up to 80 kV over a 1 M Ω resistor (Dralowid). In the present construction, a three meter long coaxial cable (Hacketal, Hannover) and single discharge is used, but in the future a 300 m long cable of the same specifications will be employed to make multiple discharge of small rectangular pulses possible. The discharge induces a signal in an aerial from the generator. The aerial is wound around the cable at the charging resistor. Its signal switches the generator off, and thus prevents a premature recharging of the cable.

The high voltage pulse is released by a movement of about 2 cm of one electrode in the spark chamber. The electrode is moved forward by compressed air (1.2 at) and controlled by a magnet valve. After the ignition of the spark, the electrode is retracted to its original position by a spring. The stationary electrode in the spark chamber is grounded over the measuring cell. As soon as the distance between the electrodes becomes smaller than the critical limit (which for air is about 1 cm), a spark of very short duration ($< 1 \mu\text{sec}$) jumps over. A current (maximally about 7 amp) then runs through the flow cell in the direction opposite to that of the liquid flow and through the lower electrode to mass. An energy of about 0.1 Joule is transferred to the solution as frictional heat. The conductivity of the solution is kept relatively high (ionic strength $\geq 0.1 M$, usually: 0.1 molar) to ensure a fast and homogeneous heating (half time $0.8 \mu\text{sec}$ at 2 mm bore of the flow channel; $3.1 \mu\text{sec}$ at 1 mm bore). The distance between the electrodes in the flow cell is so large that flow and field inhomogeneities, lense effects and complications due to electrolysis are avoided. Usually, the cable is charged to 25 kV. This voltage gives a temperature rise of about 11°C within $3 \mu\text{sec}$ with the 1 mm tube (2.7°C at 2 mm bore). The resistance of the cell is usually (0.1 molar ionic strength) 3700Ω (2 mm bore) or $15 \text{ k}\Omega$ (1 mm bore).

Optical Design (Fig. 36a). Since the primary objective of the construction of the apparatus was the investigation of chemical reactions of biological substances and since these often only are available in very small quantities, all dimensions were chosen as small as the sensitivity of the detecting system (signal/noise) permitted it (see Fig. 38). Therefore, much effort was given to achieve a good approximation to optimal design.

The lamp house can accommodate three different lamps: A quartz-iodine tungsten lamp for the range 350–700 nm (45 W, General Electric Corp.), a xenon arc lamp for 250–700 nm range (75 W, Osram XBO) and a super high pressure mercury arc lamp for as well the visible as the ultraviolet range (intense line spectrum superimposed on a weak continuous spectrum; 100 W, Osram HBO). The first two lamps are cooled by free convection through holes in the top of the lamp house, whereas the mercury lamp is cooled by air circulation from a fan mounted in a special lamp house lid.

The light is collimated by a lens which is achromatic in the range of interest and corrected for spherical aberration. The lens can travel about 10 cm along the optical axis. The beam then passes through a monochromator which is noted for its low light loss (Bausch & Lomb grating monochromator, Rochester, New York). The dispersion of the grating for the visible range is 6.4 nm/mm .

The monochromator was turned 90 degrees about its axis because the slits then became horizontal instead of vertical, which is usual. This modification of the accustomed design greatly reduced the lamp fluctuations, since the luminescent plasma of the arc almost only moved parallel to a line between the electrodes (the coaxial symmetry line). The exit slit of the monochromator was furnished with an iris diaphragm and a photographic shutter (Prontor, Gauthier, Calmbach, Schwarzwald). A system of two quartz-calcium fluoride lenses, which were corrected for chromatic and spherical aberration, made an image of the illuminated slit in the center of the flow tube. Both lenses were mounted in a tube which could be adjusted in the direction of the optical axis and vertically and horizontally with micrometer screws. The exit window of the observation cell (Fig. 36b, D) carried an aperture stop which only permitted light, which directly could strike the photocathode, to pass. The entrance window also carried an aperture stop which had an opening of a diameter equal to that of the flow tube. Hence, only light, which passed through the solution at the observation position, was allowed to enter and stray light was effectively excluded. The light beam travelled between the flow cell and the photomultiplier in a tube which excluded stray light. This tube could also be adjusted with micrometer screws.

Photoelectric equipment (Fig. 35D). The photovoltage was fed to a highly sensitive DC-differential amplifier (Textronix plug-in unit: Type D) in an oscilloscope (Textronix 545, RM 32 or storage oscilloscope type A3A). Since a single beam technique was used, it was possible to increase the sensitivity with a calibrated DC-voltage which displaced the zero line. This was done with a 9 V battery. The single beam technique was preferred, since the lamps generally were sufficiently stable for the present application, and since the considerable light loss, due to the beam splitter, was avoided. The signal-to-noise ratio was 500–1000 for the cell with the 2 mm light path.

The curve on the oscilloscope screen was photographed with a Robot camera. The pictures were then enlarged about 8 times and projected on millimeter paper for evaluation of the relaxation time.

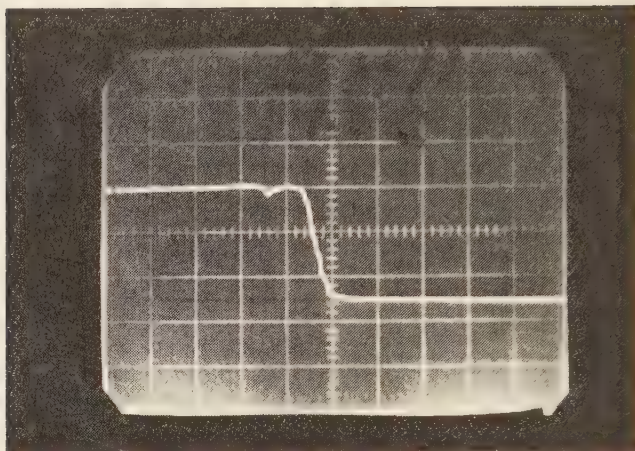


Figure 39

Mixing test (see p. 103). Solution A_I: Base + phenolphthalein solution. Solution B_I: Acid (5% excess). Ordinate: Photovoltage, 100 mV/cm. Abscissa: Time scale, 10 msec/large div.

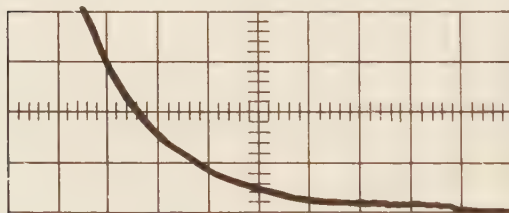


Figure 40

Photograph of a relaxation curve on the oscilloscope screen. Flow-temperature-jump experiment. Time sweep: 20 μ sec/cm. Electron transfer between p-benzoquinone and hydroquinone at pH 13 and 0.10 M ionic strength.

Tests of the efficiency of the flow-temperature-jump apparatus

Tests of the efficiency of mixing: The following systems were used to test the efficiency of the mixing of the solutions. The solutions A and B had the following composition:

- | | | |
|------|------------------|--|
| I. | A _I | 4.50 ml 1 M KOH + 1.00 ml phenolphthalein solution + 19.50 ml H ₂ O |
| | B _I | 5.00 ml 1 M HCl + 20.00 ml H ₂ O |
| II. | A _{II} | 5.00 ml 1 M KOH + 20.00 ml H ₂ O |
| | B _{II} | 4.50 ml 1 M HCl + 1.00 ml phenolphthalein solution + 19.50 ml H ₂ O |
| III. | A _{III} | 2.50 ml 1 M KNO ₃ + 22.50 ml H ₂ O |
| | B _{III} | 25.00 ml H ₂ O |

The indicator concentration was 0.05% w/w in 70% v/v aqueous ethanol.

The photography (Fig. 39), which is a typical example, shows, that the mixing is 80% complete within 4 msec when a flow tube of 2 mm bore and a velocity of 2.25 m/sec is used. The absence of fluctuations of the zero-line after passage of the gradient shows, that air bubbles, core flow, incomplete mixing and other flow imperfections were insignificant under normal conditions of operation.

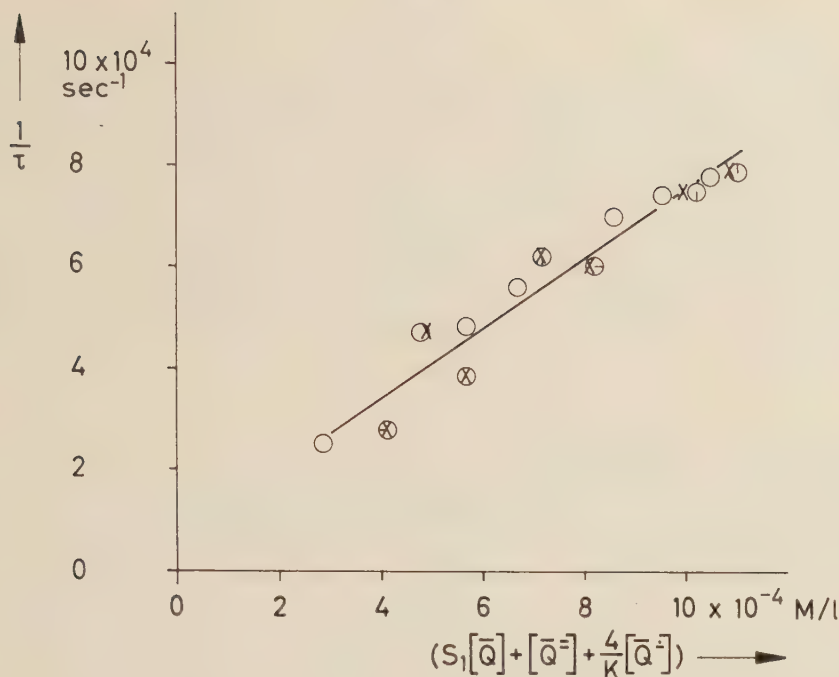


Figure 41

The interaction of p-benzoquinon (Q) with hydroquinon (Q^-) at pH 12.5 and 0.10 M ionic strength (KNO_3). The concentration dependence of the relaxation rate for electron transfer. Q^- : p-benzoquinon radical ion. The bar over the chemical symbols signifies equilibrium concentrations. Other symbols: $\circ$, 15° C, $3 \times 10^{-2} M [OH^-]$; $\odot$, 9° , $1 \times 10^{-2} M [OH^-]$; $\ominus$, 9° , $3 \times 10^{-2} M [OH^-]$; $\oplus$, 9° , $5 \times 10^{-2} M [OH^-]$; $\ominus$, 9° , $1 \times 10^{-1} M [OH^-]$; $\times$, conc. determined by direct measurement of A_{Q^-} during flow.

Tests of the linearity of the photoelectric system: The Lambert-Beer law was tested with two different systems:

- with phenolphthalein + base (without flow) and
- with the p-benzoquinonsemiradicalion (during the flow).

In both cases, a linear dependence was found between the absorbancy and the molarity. Therefore, it was concluded that aggregation did not occur in any of the two systems and that the optical and photoelectrical system produced a signal which was proportional to the arriving light flux (Fig. 42).

Test of the stopped-flow operation: The stopped-flow mode of the apparatus was tested with a reaction for which the rate constant was known. The reduction of 2,4-dichlorophenolindophenol ($5 \times 10^{-5} M$) by ascorbic acid ($1 \times 10^{-1} M$) was used. The following result was found:

$$k = (3.3 \pm 0.6) 10^7 l \times \text{Mol}^{-1} \times \text{sec}^{-1}$$

while the literature value is¹⁶⁸: $3.9 \times 10^7 l \times \text{Mol}^{-1} \times \text{sec}^{-1}$.

Test of the temperature-jump operation without flow: The instrument was tested as a temperature-jump apparatus with the electron transfer reaction between p-benzoquinon and hydroquinon at pH 11. This reaction has already been studied by Diebler and coworkers^{159, 162, 163}. Also in this case, the apparatus yielded the correct result. The data will be presented below (Fig. 41, Fig. 42).

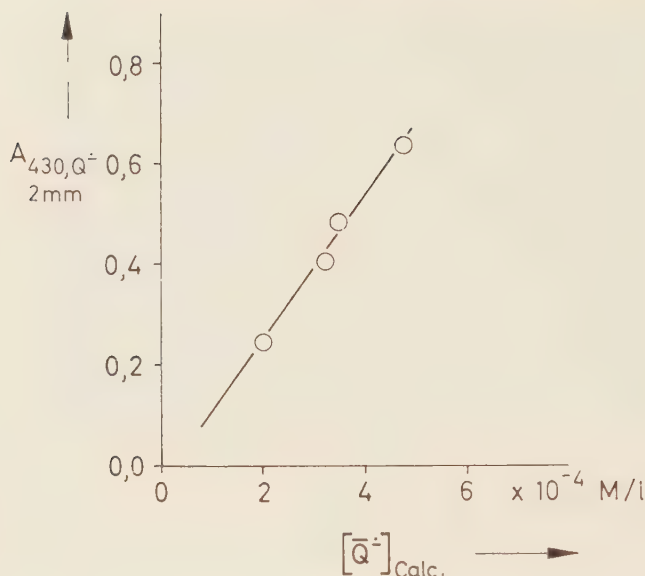


Figure 42

Test of Beer's law for the p-benzoquinon semiradical ion at pH 12.5, 9° C, during flow. $A_{430, Q^{\cdot-}}$, 2 mm; absorbancy at 430 nm in flow tube of 2 mm bore and 2 mm light path.

Test of the time resolution of the apparatus: The time resolution of the apparatus was measured with water in the observation tube at various settings of the load resistor. The curves, which due to the residual stray effect from the high-voltage discharge were displayed on the oscilloscope screen, were photographed and the apparent relaxation time (time constant for electronic recovery) was evaluated. Every curve was a very good approximation to a simple exponential function.

The tests above were confirmed with temperature-jump experiments on a phenolphthalein solution (10^{-5} M) in phosphate buffer at a pH-value near the pK' of the indicator because the chemical signal is the largest under these conditions. It is known that the chemical relaxation time of this system is much shorter than the resolution limit of the instrument.

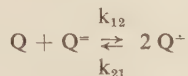
Theoretical considerations

The flow-temperature-jump apparatus can also be used for studies of reaction which are not always progressing under equilibrium conditions. Previously, almost all kinetic experiments bearing on enzyme catalysis have been performed at equilibrium or quasi-equilibrium, usually in the steady state. Since the present instrument is not limited in this respect, new applications, which include open systems and many fast unbalanced reactions, are now possible.

The kinetic theory for the perturbation of chemical system in or near equilibrium has already been developed by Eigen, DeMaeyer¹⁶⁰, Meixner¹⁶¹, Jost¹⁶¹ and others. Therefore, the application of the relaxation theory to flow systems in equilibrium should not pose any new difficulties. However, if the reaction oscillates, then a transient perturbation will have an influence which previously has not been treated theoretically. Such a study would exceed the limits of this dissertation. No simple reaction showing oscillations is so far known, but examples will probably soon be found, since much effort now is being given to analysis of complex, oscillating, biological reactions.

Conclusions

1. A combined flow- and temperature-jump method has been developed. The principle is the perturbation of a flowing system in the steady-state by a single, almost rectangular temperature-jump. Previously, only equilibrium systems at rest have been investigated. The instrument can also be used as a constant-flow-, a stopped-flow- and a micro-temperature-jump-apparatus.
2. Tests of the efficiency of the various modes of operation of the apparatus. Beer's law is accurately obeyed for the p-benzoquinon-semiradical ion in the concentration range used ($(2 - 10) \times 10^{-4}$ molar). This proves, that the response of the photoelectric systems to the light flux is linear. Besides, the ion must almost exclusively be present as a monomer, since the kinetic data appear to exclude reaction schemes involving aggregation.
3. The rate constant for the electron transfer between p-benzoquinon (Q) and hydroquinon ($Q^{\cdot-}$) at pH 12.5 in the forward direction, k_{12} , was measured (Fig. 40, 41):



$k_{12} = (0.69 \pm 0.01) \times 10^8 \text{ l} \times \text{Mol}^{-1} \times \text{sec}^{-1}$ was found. This value is in reasonable agreement with the earlier measurements at lower pH. The pH-dependence of this rate constant was found to be slight and the activation energy was very small.

4. Flexibility of operation, good fluid economy and a mild treatment of the reactants was emphasized in the development of the method, since kinetic studies of enzyme-catalyzed reactions were expected to be its main application. The instrument can, however, be used for studies of the kinetics of almost any reaction in solution.

Chapter 7

Dansk Resumé

Kapitel I. Formålet med undersøgelserne i den foreliggende afhandling er at give et bidrag til forståelsen af de strukturelle ændringer, der indtræder i enzymer i forbindelse med deres katalytiske virkning. Sådanne undersøgelser er nødvendige for opnåelsen af en dybere forståelse af selve mekanismen bag den katalytiske proces.

Baggrunden for de udførte undersøgelser er beskrevet i hovedtræk i kapitel I, der munder ud i en oversigt over forfatterens tidligere bidrag på dette område. De er en direkte forudsætning for arbejdet i denne afhandling. Et rationelt studium af betydningen af konformationsændringerne i enzymer må baseres på samhørende undersøgelser af disse proteins statiske (strukturelle) og dynamiske (reaktionskinetiske) egenskaber under omdannelse af et specifikt substrat. Derved opnås den bedst mulige udnyttelse af de eksperimentelle resultater, som hver for sig ofte er utilstrækkelige for en fuldstændig bevisførelse, fordi de udsagn, som en foreliggende observation tillader, oftest kun er et indirekte bevis for den opstillede hypotheses rigtighed. En samtidig måling af flere beslægtede fysiske egenskaber af enzymet med metoder, der er gensidigt uafhængige, giver imidlertid resultater, der yder hinanden en betydelig støtte. De enkelte konklusioner, der kan drages fra hver type af observationer, kan derfor sammenfattes til en helhed. Rigtigheden af de slutninger, der blev draget på dette grundlag, er senere blevet bekræftet ved direkte metoder, der først er blevet udviklet for nylig.

Da planen for projektet, der førte til denne afhandling, blev udarbejdet, var få enzymer blevet undersøgt så indgående som chymotrypsin. Derfor valgte forfatteren også at gøre dette enzym til genstand for den grundigste undersøgelse under studiet af detaillerne og betydningen af konformationelle ændringer. De nye resultater kan betragtes som et supplement til forfatterens tidligere afhandlinger over enzymkatalyse.

Kapitel II. Literaturoversigt. Referatet af den omfattende litteratur om emnet er begrænset til de arbejder, som forfatteren finder har størst betydning.

Kapitel III. Denne del af afhandlingen omfatter de eksperimentelle undersøgelser. Disse suppleres med et appendix, der omhandler udførelsen af en mere speciel opgave, der ikke har direkte relation til kapitel III, men som er nødvendig for den logiske videreførelse af projektet.

3.1.1. Kapitlets første afsnit omhandler elektrometriske titreringer af α -chymotrypsin (α -CT) og diisopropyl-phosphoryl-chymotrypsin (DIP-CT).

Formålet var her at konstatere om dannelsen af DIP-CT, som er en intermediær forbindelse i chymotrypsinets hydrolyse af substratet diisopropylfluoro-phosphat (DFP), fører til ændringer i pK-værdierne for de af aminosyresidekæderne, der deltager i protolytiske reaktioner.

Titrationerne udførtes i pH-området 2.5–11.5 i 0.15 molær KCl-opløsning ved 4, 16 og 25° C (ved 25° C titreredes til pH 1.5). Eksperimentet blev gentaget i 0.40 molær KCl ved 4° og 16° C og i 8 molær urinstof ved 25° C. Kurverne var reversible i pH-området 3.5–11.5. I alle forsøgene fandtes omtrent den samme differensititreringskurve mellem CT og DIP-CT ved pH 8.5–10.5 Denne effekt vedvarede efter to timers behandling i otte molær urinstofopløsning ved pH 6 og stuetemperatur.

De to enzymer (CT og DIP-CT) har hver tretten karboxylgrupper. Alle disse viste sig at have samme »intrinsiske« pK-værdi, som var usædvanlig lav. Derimod var de elektrostatiske faktorer, w , forskellige for de to enzymer. Dette kan skyldes, at enzymet har fået en ekstra positiv ladning (α -NH₃⁺) ved reaktionen med DFP.

3.1.2. De kinetiske studier af chymotrypsinets reaktioner indledtes med en specificitetsundersøgelse. Dertil anvendtes en såkaldt kemisk relaxationsmetode. Den teknik, der var til rådighed på dette stadium, beroede på en hurtig temperaturforandring i et lukket system. Denne metode tillod ikke undersøgelse af reaktioner med forskudte ligevægte. Derfor måtte substraterne erstattes med beslægtede kompetitive inhibitorer, der tillige var farvestoffer. Herved udelukkedes frigørelsen og ansamlingen af produkter. Hastighedskonstanterne for elementærtrinnene ved inhibitorernes reaktion med CT kunne bestemmes for flere af disse substratmodeller.

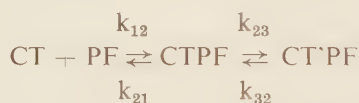
Specificitetsundersøgelsen viste, at de aromatiske forbindelser, der optrådte som modeller af specifikke CT-substrater, havde en udpræget tendens til polymerisering (især dimerisering). Dette fænomen må spille en stor rolle i den katalytiske reaktions kinetik, når inhibitor-enzym-molforholdet er af betydelig størrelse. Spektrofotometriske og kinetiske metoder, der beroede på, at enzymet forskød polymerisationsligevægten i retning af depolymerisation, blev anvendt. Denne virkning var især tydelig for acridin orange. Den nævnte polymerisering er langsommere end, eller forløber med omtrent samme hastighed som, den egentlige substrat-enzym-reaktion. De forbindelser, hvor acridinkernen bærer en fri aminogruppe i stillingerne 3 og 6 (proflavin og acriflavin) er undtagne herfra. Når disse aminogrupeer alkyleres med to metylgrupper hver (som i acridin orange), tager farvestoffernes gensidige vekselvirkning atter overhånd. *Den spektrofotometriske titrering og kinetikken viser, at absorptionen af acridin orange til chymotrypsin ved pH 6.7 ledsages af en omlejring i inhibitor-enzym-komplekset.*

Alkylering af proflavin (til acridin orange) fører kun til en mindre ændring i absorptionsspektret. Det bekræfter formodningen om, at polymeriseringen hovedsagelig skyldes såkaldte hydrophobe vekselvirkninger, der beror på ændringer i

den ordnede vandstruktur om molekylets upolære dele ved kompleksdannelse. Da det vides, at acridinderivaternes biologiske virkning er stærkt afhængig af tilstedeværelsen af en positiv ladning på kvælstofatomet i den aromatiske ring, er det sandsynligt, at jonbinding er af betydning for inhibitorernes kompleksdannelse med enzymer som CT og trypsin.

Inhibitorerne fremkaldte (med undtagelse af acridin orange) kun en enkelt relaxationseffekt ved pH 6.7 i enzymets tilstedeværelse. Reaktionen er derfor et simpelt adsorptionsfænomen under de anførte omstændigheder. Derimod opstod flere relaxationseffekter ved udførelsen af et temperaturspringeksperiment med systemerne chymotrypsin-proflavin, -acriflavin og -acridin orange ved pH 9.2 og 9.6. Disse iagttagelser kunne kun forklares ved antagelsen af tilstedeværelsen af to på hinanden følgende reaktionstrin, en kompleksdannelse efterfulgt af en omlejring af komplekset. *Dette resultat yder en betydelig støtte til den opstillede hypotese om, at chymotrypsinets og trypsinets katalyse ledsages af konformationelle ændringer i enzymet.*

3.1.3. Derefter foretog forfatteren en nærmere undersøgelse af de kinetiske parametres pH-afhængighed ved chymotrypsinets vekselvirkning med en kompetitiv inhibitor. Proflavin (PF) valgtes hertil på grund af sine gunstige optiske egenskaber, og fordi dets kompleksitetskonstant for chymotrypsin er omtrent identisk med bindingskonstanten for et typisk substrat af dette enzym. Reaktionen mellem CT og PF kunne beskrives som en sekvens af to trin:



Alle andre reaktionsveje, som forfatteren kunne opstille, var i uoverensstemmelse med de observerede hastighedslove. Samtlige hastighedskonstanter i det opstillede kinetiske skema kunne beregnes uden andre antagelser end, at reaktionen var stærkt forskudt til venstre for den bratte temperaturstigning. Dette synspunkt blev understøttet af den direkte iagttagelse af ligevægten: $\text{CT}_1 \rightleftharpoons \text{CT}$ ved pH 9 i inhibitorens fravær. Disse eksperimenter udførtes med et temperaturspringsapparat med polarisationsoptik. Ligevægtskonstanterne, der beregnedes ud fra hastighedskonstanterne er i rimelig overensstemmelse med de, som blev bestemt ved spektrophotometrisk titrering, ligevægtsdialyse og anslået ved sammenligning med hæmningskonstanter.

Kinetiske ligninger, der tilfredsstiller de eksperimentelle resultater for hastighedskonstanternes pH-afhængighed i pH-området 6.7–10.3, blev opstillet. Heraf udledtes et kinetisk skema for de vigtigste vekselvirkninger i det alkaliske pH-område mellem chymotrypsin og den kompetitive inhibitor.

Proflavinets vekselvirkning med trypsin ved pH 8.8 blev ligeledes undersøgt. Dette enzym synes under disse forsøgsomstændigheder at forholde sig på omtrent samme måde som chymotrypsin.

3.2. Da det indsamlede materiale til støtte for hypotesen om de konformationelle ændringer i hydrolaser som chymotrypsin og trypsin var blevet så omfattende, at den accepteredes af de fleste af feltets specialister, koncentreredes arbejdet om en undersøgelse af, om fænomenet var alment for enzymkatalyse eller blot karakteristisk for visse hydrolaser. Forfatteren valgte glyceraldehyd-3-phosphat-dehydrogenase (G3PDH) til denne undersøgelse, fordi dette enzym indtager en vigtig stilling i glykolysen, og fordi mange af dets kemiske egenskaber er kendte. Dette enzym er også interessant, fordi det udøver flere forskellige katalytiske funktioner. Det katalyserer således såvel elektronovergang som fosforolyse og hydrolytisk esterspaltning. Desuden repræsenterer G3PDH det første veldokumenterede eksempel på allosterisk kontrol.

Enzymets symmetri og andre strukturelle egenskaber undersøgte i tilstedeværelse af og i fravær af cofaktoren nicotin-adenin-dinucleotid (NAD) og substratet glyceraldehyd-3-phosphat (G3P) ved måling af den optiske rotationsdispersion (O.R.D.) i området 300–700 nm. Endvidere udførtes foreløbige eksperimenter med et isoleret mellemprodukt i den katalytiske reaktion, 3-phosphoglycerat thiolester-G3PDH.

Resultatet af undersøgelsen var, at det tilsyneladende indhold af skrueformede strukturer (helices) i molekylet var afhængigt af pH i området pH 7.7–8.4. Det var O.R.D.-parameteren K , der anses for at repræsentere det asymmetriske centrumssolvatisering (polaritet), derimod ikke. Binding af stoikiometriske mængder af NAD til enzymets proteindiel (apoenzymet) ændrede O.R.D.-parameteren b_0 , der repræsenterer enzymets indhold af helices, men ikke det asymmetriske centrumssolvatisering. Tilstedeværelsen af et overskud af NAD forårsagede ingen yderligere ændringer i O.R.D.-kurven udover en mindre forøgelse af den anormale rotationsamplitude ved 650 nm.

En særlig undersøgelse foretoges af aktivatoren α -thioglycerols indflydelse på apoenzymets optiske rotationsdispersion. Selv en lav koncentration (6×10^{-4} molær) af denne upolære forbindelse, ændrede O.R.D.-parametrene a_0 og b_0 mærkbart. *Aktivatorens virkning kan derfor, i hvert fald delvis, bero på en kompleksdannelse, der udløser en konformationsændring i enzymet.*

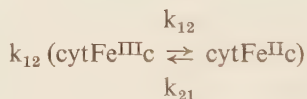
Dannelsen af glyceraldehyd-3-phosphat-hemimerkaptal-enzymet fra apoenzymet ledsagedes af ændringer i såvel b_0 som K . Endvidere opstod en anormal rotation i to bølgelængdeområder: nær 305 nm og nær 650 nm. En sammenligning med modelsystemers optiske rotationsdispersion viser, at effekten ved 305 nm kunne skyldes en Cotton-effekt af et Zinkkompleks. Den anden anomali hidrører muligvis fra en vekselvirkning med svovlatomet i cystein. Denne formodning støttes af den iagttagelse, at visse divalente metaljoners komplekser med cystein har absorptionsbånd i området 580–700 nm. Det er endvidere velkendt, at enzymets aktive område besidder en sulfhydrylgruppe, der er essentiel for den biologiske aktivitet, og som derfor deltager i dannelsen af G3P-hemimerkaptalenzymet.

3.3. Undersøgelsen af de katalytiske proteiners mekanisme blev derefter udvidet med studier af elektronovergangsreaktioner i respirationskæden, idet det for-

ventedes, at også denne proces kunne ledsages af konformationsændringer i katalysatoren. Forfatteren valgte cytochrom c til denne opgave, fordi dette protein er det mest stabile, lettest tilgængelige og bedst kendte af de respiratoriske pigmenter. Cytochrom c omfattes endvidere med en særlig interesse, fordi dette led i respirationskæden er det langsomste og derfor det hastighedsbestemmende. Cytoferrat-systemet valgtes som reaktionspartner for cytochrom c, da cytoferrat-komplekset havde en symmetri, der var nært beslægtet med den, der karakteriserede cytochromernes h  m-del. Derfor m   det formodes, at ret vidtstrakte analogier fra modelsystemet til forholdene i respirationsk  den er tilladelige. Elektronovergangens kinetik blev unders  gt ved hj  lp af temperaturspringsmetoden, da denne teknik tillader m  ling af de store reaktionshastigheder og en udredning af den komplekse mekanisme.

Elektronovergangen mellem ferricytochrom c og kaliumferrocyanid fandtes at v  re ret langsom for en reaktion af denne type, men den var langt hurtigere end konformations  ndringerne. De f  rste forl  ber i millisekundomr  det, mens isomeriseringen er tre st  rrelsesordner langsommere. Disse resultater er i overensstemmelse med tidligere rapporter om, at ferricytochrom c kan eksistere i to former, hvoraf kun een deltager i den katalytiske reaktion i n  vnev  rdig grad.

Hastighedskonstanten:



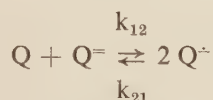
udviste en pH-afh  ngighed, der i hovedsagen fulgte den protolytiske titreringskurve (og normalpotentialet E^0). Specifikke effekter af betydelig st  rrelse fandtes imidlertid n  r pH 7.1, 9.0 og 4.8. Ved de to f  rste pH-v  rdier var hastigheden for  get, mens den ved pH 4.8 var formindsket. Det er sandsynligt, at disse f  n  mener skyldtes par af joniserende grupper med pK-v  rdier n  r de angivne ekstreme v  rdier for aktiviteten. S  danne protolytiske grupper tilh  rer muligvis to h  m-bundne histidinrester, to imidazolsidek  der, der ikke er bundne til h  m, og to karboxylgrupper.

Appendix. I dette afsnit beskrives et arbejde, der er af en noget anden natur end det foreg  ende, men som er en konsekvent videref  relse af unders  gelsen af enzymernes reaktionskinetik. De hidtidige metoder tillod kun m  ling af hurtige reaktioner, hvis ligev  gte omtrent var i balance, eller af ret langsomme ubalancerede reaktioner (indtil millisekundomr  det). Hydrolasernes katalyse, som er hovedtemaet i denne afhandling, er imidlertid karakteriseret ved tilstedev  relsen af meget hurtige, ubalancerede reaktioner. Da disse rummer n  glen til vigtige, og formodentlig de eneste endnu ukendte sider af enzymkatalysens mekanisme, besluttede forfatteren at konstruere et nyt instrument, et kombineret str  mnings- og temperaturspringsapparat, som skulle kunne anvendes til l  sning af dette problem. Denne antagelse er senere blevet bekr  ftet.

Princippet i den nye metode er en forstyrrelse af et strømmende system, der befinder sig i en stationær tilstand, med en enkelt, hurtig og tilnærmelsesvis diskontinuerlig temperaturstigning. De kemiske ligevægte vil derefter indstille sig under de nye betingelser med en hastighed, hvis koncentrationsafhængighed tillader beregning af elementærskridtenes hastighedskonstanter. Instrumentet kan tillige anvendes til eksperimenter med konstant strømning, afbrudt strømning og temperaturspring med mikromængder i et hydrodynamisk hvilende system.

Det nye apparats fotoelektriske system blev afprøvet med et stabilt farvestof (phenolphthalein i puffer) og med et metastabil radikal (p-benzosemiquinon-radikalion, $Q^{\cdot-}$). I begge tilfælde var Beers lov nøje opfyldt i det anvendte koncentrationsinterval ($(2 - 10) \times 10^{-4}$ molær for $Q^{\cdot-}$). Dette beviser, at registreringsanordningen er tilfredsstillende og støtter formodningen om, at radikalionen er til stede i monomer form. Det sidste bekræftes af de kinetiske målinger, som udelukker, at simpel polymerisering spiller nogen rolle af betydning.

Hele anlægget blev afprøvet med reaktionen mellem p-benzoquinon (Q) og hydroquinon($Q^=$) ved pH 12.5:



Hastighedskonstanten k_{12} for denne elektronovergangsreaktion fandtes at være $(0.69 \pm 0.01) \times 10^8 \text{ l} \times \text{mol}^{-1} \text{ sec}^{-1}$. Denne værdi er i tilstrækkelig overensstemmelse med tidligere målinger.

Ved apparatets konstruktion blev der især lagt vægt på, at det skulle anvendes til forskelligartede eksperimenter, på en god økonomi med de biologiske materialer og på en skånsom behandling af disse. Instrumentets hovedanvendelse forventes at blive kinetiske studier af reaktioner som katalyseres af enzymer, men det kan anvendes til studier af næsten enhver reaktion i opløsning.

Kapitel IV. Kapitlet indledes med en oversigt over det stade, som opklaringen af de konformationelle ændringer i enzymer har nået. Der lægges især vægt på chymotrypsinets forhold, da dette enzym på tidspunktet for manuskriptets affattelse repræsenterede det bedst kendte eksempel. Derefter diskuteres afhandlingens eksperimentelle afsnit og de dertil knyttede almene problemer.

Kapitel V. Afhandlingen afsluttes med de almene konklusioner vedrørende konformationsændringer i enzymer, som forfatteren mener at kunne drage. Der gives endvidere en oversigt over de muligheder, der foreligger for en videreførelse af projektet.

Disputatsens væsentligste konklusion er, at mange katalytiske proteiner (muligvis alle) undergår omlejninger under deres virksomhed. Dette fænomen menes at være betydningsfuldt for deres fysiologiske virkning. Det anføres endvidere, at der intet principielt er til hinder for, at hvert enkelt trin i enzymernes reaktionsmekanisme kan måles og beskrives indgående.

Afhandlingen afsluttes med en henvisning til, at der foreligger et ret anseligt materiale, der tyder på, at visse generelle love gælder for de forskellige enzyms katalytiske virkning og specielt for konformationsændringernes natur. Disse love beskrives i hovedtræk. Hvis formodningen om, at der er en udstrakt lighed mellem de forskellige enzyms fundamentale principper, er rigtig, er en vigtig erkendelse i opklaringen af sammenhængen mellem den kemiske struktur og den biologiske virkning opnået.

Bibliography

1. Boyer, P. D., Lardy H. and Myrbäck, K., eds., "The Enzymes", 2nd. ed., Academic Press 1959, New York.
2. Smith, E. L., *Advances in Enzymology*, **12**, 195 (1951).
3. Anfinsen, C. B., Haber, E., Sela, M. and White, F. H., Jr., *Proc. Nat. Acad. Sci., US*, **47**, 1309 (1961).
4. Labouesse, B., Havsteen, B. H. and Hess, G. P., *Proc. Nat. Acad. Sci., US*, **48**, 2137 (1962).
5. Anfinsen, C. B., "The Molecular Basis of Evolution". Wiley, New York, 1959. p. 136.
6. Némethy, G. and Scheraga, H. A., *J. Phys. Chem.*, **66**, 1773 (1962).
7. Perutz, M. F., Kendrew, J. C. and Watson, H. C., *J. Mol. Biol.*, **13**, 669 (1965).
8. Havsteen, B. H. and Hess, G. P., *J. Am. Chem. Soc.*, **84**, 491 (1962).
9. Tanford, C., *J. Am. Chem. Soc.*, **84**, 4240 (1962).
10. Neurath, H., ed., "The Proteins", 3rd ed., Academic Press, 1965, New York. Vol. III, p. 445.
11. Changeux, J.-P., in "Brookhaven Symposia in Biology", no. 17, 1964, p. 232.
12. Kirschner, K., Bittman, R. and Voigt, B., *Proc. Nat. Acad. Sci.*, **56**, 1661 (1966).
13. Gerhart, J. C., in "Brookhaven Symposia in Biology", no. 17, 1964, p. 222.
14. Schuster, T. M., Ilgenfritz, G., Eigen, M. and Brunori, M., *Abstr. 152th Meeting of the American Chemical Society*, New York. Sept., 1966.
15. Euler, von H. and Josephson, K., *Z. Physiol. Chem.*, **157**, 122 (1926).
16. Bergmann, M., "Harvey Lectures", **31**, 37 (1935-36).
17. Eyring, H., Lumry, R. and Spikes, J. D. in: "The Mechanism of Enzyme Action", McElroy, W. D. and Glass, B., eds., Johns Hopkins Univ. Press, Baltimore, 1954, p. 132.
18. Smith, E. L., *Advances in Enzymology*, **12**, 191 (1951).
19. Johnson, F. H., *Advances in Enzymology*, **7**, 237 (1947).
20. Laidler, K. J., *Arch. Biochem.*, **30**, 226 (1951), *J. Am. Chem. Soc.*, **72**, 2489 (1950).
21. Vaslow, F. and Doherty, D. G., *J. Am. Chem. Soc.*, **75**, 928 (1953).
22. Vaslow, F., *Compt. Rend. Trav. Lab. Carlsberg (Ser. Chim.)*, **31**, 29 (1960).
23. Scheraga, H. A., Némethy, G. and Steinberg, I. Z., *J. Biol. Chem.*, **237**, 2506 (1962).
24. Linderstrøm-Lang, K. U. and Schellman, J. A. in: "The Enzymes", 1st. ed., Boyer, P. D., Lardy, H. and Myrbäck, K., eds., Academic Press, New York, Vol. I, 1959, p. 472.
25. Thoma, J. A. and Koshland, Jr., D. E., *J. Am. Chem. Soc.*, **82**, 3329 (1960).
26. Moon, A. Y., Sturtevant, J. M. and Hess, G. P., *Fed. Proc.*, **21**, 229 (1962).
27. Moon, A. Y., Mercouroff, J. and Hess, G. P., *J. Biol. Chem.*, **240**, 717 (1965).
28. Cohen, W. and Erlanger, B. F., *J. Am. Chem. Soc.*, **82**, 3928 (1960).
29. Szabolcsi, G. and Biszku, E., *Biochim. Biophys. Acta*, **48**, 335 (1961).
30. Kunitz, M. and McDonald, M. R., *J. Gen. Physiol.*, **29**, 393 (1946).
31. Barnard, E. A., *Biochem. J.*, **83**, 14P (1962).
32. Citri, N. and Garber, N., *Biochem. Biophys. Res. Comm.*, **4**, 143 (1961).
33. Neurath, H., Rupley, J. A. and Dreyer, W. J., *Arch. Biochem. Biophys.*, **65**, 243 (1956).
34. Lumry, R. and Parker, H., *Fed. Proc.*, **21**, 246 (1962).
35. Samuels, A. J., Nihei, T. and Noda, L., *Proc. Nat. Acad. Sci., US*, **47**, 1992 (1961).
36. Yagi, K., Ozawa, T. and Ooi, T., *Biochim. Biophys. Acta*, **54**, 199 (1961).
37. Yagi, K. and Ozawa, T., *ibid.*, **56**, 413 (1962).
38. Yagi, K., Ozawa, T. and Ooi, T., *ibid.*, **77**, 20 (1963).
39. Yagi, K., Ozawa, T. and Harada, M., *Nature*, **188**, 745 (1960).
40. Wootton, J. F. and Hess, G. P., *J. Am. Chem. Soc.*, **84**, 440 (1962).

41. Williams, E. J. and Laskowski, Jr., M., Abstr. 141st Nat. Meeting of the American Chemical Society, Washington, DC, 1962, p. 42 c. Williams, E. J., Herskovits, T. T. and Laskowski, Jr., M., *J. Biol. Chem.*, **240**, 3574 (1965).
42. Oppenheimer, H. L., Mercouroff, J. and Hess, G. P., *Biochim. Biophys. Acta*, **71**, 78 (1963).
43. Biltonen, R., Lumry, R., Madison, V. and Parker, H., *Proc. Nat. Acad. Sci., US*, **54**, 1412 (1965).
44. Bruice, T. C., *Proc. Nat. Acad. Sci., US*, **47**, 1924 (1961).
45. Platt, A. and Niemann, C., *Proc. Nat. Acad. Sci., US*, **50**, 817 (1963).
46. Oppenheimer, H. L., Labouesse, B. and Hess, G. P., *J. Biol. Chem.*, **241**, 2720 (1966).
47. Sigler, P. B. and Skinner, H. C. W., *Biochem. Biophys. Res. Comm.*, **13**, 236 (1963).
48. Blow, D. M., Rossmann, M. G. and Jeffery, B. A., *J. Mol. Biol.*, **8**, 65 (1964).
49. Johnson, L. M. and Phillips, D. C., *Nature*, **206**, 761 (1965). Phillips, D. C., *Scientific American*, **215**, 78 (1966). (No. 5).
50. Doscher, M. S. and Richards, F. M., *J. Biol. Chem.*, **238**, 2399 (1963).
51. Kendrew, J. C., in "Brookhaven Symposium in Biology", no. 15, 216 (1962) *Ber. Bunsenges. Phys., Chem.*, **68**, 721 (1964).
52. Praissman, M. and Rupley, J. A., *J. Am. Chem. Soc.*, **86**, 3584 (1964).
53. Schachter, H., Halliday, K. A. and Dixon, G. H., *J. Biol. Chem.*, **238**, PC 3134 (1963).
54. Matthews, B. W., Sigler, P. B., Henderson, R. and Blow, D. M., *Nature*, **214**, 652 (1967).
55. Hvidt, Å., Kågi, J. H. R. and Ottesen, M., *Biochim. Biophys. Acta*, **75**, 290 (1963).
56. Hvidt, Å. and Kågi, J. H. R., *Compt. Rend. Lab. Carlsberg, Ser. Chim.*, **33**, 497 (1963).
57. Hayashi, K., Imoto, T. and Funatsu, M., *J. Biochem. (Tokyo)*, **54**, 381 (1963).
58. Havsteen, B. H. and Hess, G. P., *Biochem. Biophys. Res. Comm.*, **14**, 313 (1964).
59. Nakamura, S., Takeo, K. and Sasaki, I., *Z. Physiol. Chem.*, **334**, 95 (1963).
60. Weiner, H. and Koshland, Jr., D. E., *J. Mol. Biol.*, **12**, 881 (1965).
61. Hammes, G. G., *Nature (London)*, **204**, 342 (1964).
62. Bernhard, S. A. and Gutfreund, H., *Proc. Nat. Acad. Sci., US*, **53**, 1238 (1965).
63. Barman, T. E. and Gutfreund, H., *ibid.*, **53**, 1243 (1965).
64. Barman, T. E. and Gutfreund, H., *Biochem. J.*, **101**, 411 (1966).
65. Bender, M. L., Schonbaum, G. R. and Zerner, B., *J. Am. Chem. Soc.*, **84**, 2540 (1962).
66. Wilson, I. B. in "Mechanism of Enzyme Action", W. D. McElroy and B. Glass, eds., Johns Hopkins University Press, Baltimore, 1954, p. 653.
67. Greenwood, C. and Palmer, G., *J. Biol. Chem.*, **240**, 3660 (1965).
68. Brandt, K. G., Parks, P. C., Czerlinski, G. H. and Hess, G. P., *J. Biol. Chem.*, **241**, 4180, (1966).
69. Schaffer, N. K., May, S. C., Jr. and Summerson, W. H., *J. Biol. Chem.*, **202**, 67 (1953).
70. Crammer, J. L. and Neuberger, A., *Biochem. J.*, **37**, 302 (1943).
71. Havsteen, B. H. and Hess, G. P., *J. Am. Chem. Soc.*, **84**, 448 (1962).
72. Heller, W., in "Physical Methods of Organic Chemistry", Weissberger, A., ed.; Interscience Publ., New York, 2nd. Ed., part II, 1949. p. 1506. Leach, S. J. and Scheraga, H. A., *J. Am. Chem. Soc.*, **82**, 4790 (1960).
73. Blout, E. R., De Lozé, C. and Asadourian, A., *J. Am. Chem. Soc.*, **83**, 1895 (1961). Urnes, P. and Doty, P., *Advances in Protein Chem.*, **16**, 401 (1961).
74. Labouesse, B., Carlsson, K., Oppenheimer, H. L. and Hess, G. P., in "Structure and Activity of Enzymes", Goodwin, T. W., Harris J. I. and Hartley, B., eds., Academic Press, London, 1964, p. 71.
75. Linderstrøm-Lang, K. U., *Compt. Rend. Lab. Carlsberg, Ser. Chim.*, **15**, no. 7 (1924).
76. Edsall, J. T. and Wyman, J., "Biophysical Chemistry", 1958, Academic Press, New York, Vol. I, Ch. 9.
77. Tanford, C. and Kirkwood, J. G., *J. Am. Chem. Soc.*, **79**, 5333 (1957).
78. Tanford, C. in "Electrochemistry in Biology and Medicine", T. Shedlovsky, ed., John Wiley & Sons, Inc., New York, 1955, p. 248.
79. Scheraga, H. A., "Protein Structure", Academic Press, New York, 1961, p. 254.
80. Marini, M. and Wunsch, C., *Biochem.*, **2**, 1454 (1963), *Anal. Biochem.*, **6**, 534 (1963).
81. Jansen, E. F., Nutting, M.-D. F., Jang, R. and Balls, A. K., *J. Biol. Chem.*, **185**, 209 (1950).
82. Hartley, B., in "Structure and Activity of Enzymes", Goodwin, T. W., Harris, J. I. and Hartley, B., eds., Academic Press, London, 1964, p. 47.

83. Sumner, J. B., *Science*, **100**, 413 (1944).
84. Kolthoff, J. M., *Z. Anal. Chem.*, **61**, 48 (1922).
85. Bates, R. G., "Electrometric pH-Determination", John Wiley & Sons, Inc., New York, 1954, p. 118.
86. Anderson, E. A. and Alberty, R. A., *J. Phys. & Col. Chem.*, **52**, 1345 (1948).
87. Scatchard, G., Batchelder, A. C. and Brown, A., *J. Am. Chem. Soc.*, **68**, 2320 (1946).
88. Hartley, B. S., *J. Cell. Comp. Physiol.*, **54**, Suppl. 1, 203 (1959).
89. Wallace, R. A., Kurtz, A. N. and Niemann, C., *Biochem.*, **2**, 824 (1963).
90. Wallace, R. A., Peterson, R. L., Niemann, C. and Hein, G. E., *Biochem. Biophys. Res. Comm.*, **23**, 246 (1966).
91. Bernhard, S. A. and Lee, B. F., *Abstr. VIth Intern. Congr. Biochem.*, New York (1964), IUB Vol. **32**, p. 297, IV-9.
92. Brandt, K. G. and Hess, G. P., *Biochem. Biophys. Res. Comm.*, **22**, 447 (1966).
93. Glazer, A. N., *Proc. Nat. Acad. Sci., US*, **54**, 171 (1965).
94. Schwert, G. W., Neurath, H., Kaufman, S. and Snoke, J. E., *J. Biol. Chem.*, **172**, 221 (1948).
95. Czerlinski, G. and Eigen, M., *Z. Electrochem.*, **63**, 652 (1959).
96. Eigen, M. and DeMaeyer, L., in "Techniques of Organic Chemistry", Friess, S. L., Lewis, E. S. and Weissberger, A. eds., Interscience, London, 1963, Vol. VIII, part 2, p. 895.
97. Yapel, A. and Lumry, R., *J. Am. Chem. Soc.*, **86**, 4499 (1964).
98. Schneider, F. and Podder, S., in preparation, 1966.
99. Zanker, V., *Z. physik. Chem.*, **199**, 225 (1951).
100. Haugen, G. R. and Melhuish, W. H., *Trans. Farad. Soc.*, **60**, 386 (1964).
101. Sigler, P. B., Jeffery, B. A., Matthews, B. W. and Blow, D. M., *J. Mol. Biol.*, **15**, 175 (1966).
102. Kunitz, M., *J. Gen. Physiol.*, **30**, 291 (1947).
103. Eigen, M. and Hammes, G. G., *Advances in Enzymology*, **25**, 1 (1963).
104. Velick, S. F. and Furfine, C., in "The Enzymes", Boyer, P. D., Lardy, H. and Myrbäck, K., eds., Academic Press, 1963, Vol. 7, p. 243.
105. Krimsky, I. and Racker, E., *Science*, **122**, 319 (1955); *Biochem.*, **2**, 512 (1963).
106. Cori, G. T., Slein, M. W. and Cori, C. F., *J. Biol. Chem.*, **173**, 605 (1948).
107. Harris, I., Meriwether, B. P. and Park, J. H., *Nature*, **198**, 154 (1963).
108. Segal, H. L. and Gold, A. H., *J. Biol. Chem.*, **238**, PC 2589 (1963).
109. Hilvers, A. G., van Dam, K. and Slater, E. C., *Biochim. Biophys. Acta.*, **85**, 206 (1964).
110. Listowsky, I., Furfine, C. S., Bethell, J. J. and England, S., *J. Biol. Chem.*, **240**, 4253 (1965).
111. Adair, G. S., *J. Biol. Chem.*, **63**, 529 (1925).
112. Boyer, P. D. and Segal, H. L., in "The Mechanism of Enzyme Action", McElroy, W. D., and Glass, B., eds., Johns Hopkins University Press, Baltimore, 1954, p. 520.
113. Boyer, P. D., in "Sulfur in Proteins", Benesch, R. et al., eds., Academic Press, New York, 1959, p. 211.
114. Elödi, P. and Szabolcsi, G., *Nature*, **184**, 56 (1959).
115. Krebs, E. G., in "Methods of Enzymology", Kaplan, N. O. and Colowick, S. P., eds., 1st ed., Academic Press, New York, 1955, Vol. I, p. 410.
116. Fox, Jr., J. and Dandliker, W. B., *J. Biol. Chem.*, **221**, 1005 (1956).
117. Dandliker, W. B. and Fox, Jr., J. B., *ibid.*, **214**, 275 (1955).
118. Wu, R. and Racker, E., *J. Biol. Chem.*, **234**, 1029 (1959).
119. Racker, E., Klybas, V. and Schramm, M., *J. Biol. Chem.*, **234**, 2510 (1959).
120. Bartlett, G. R., *J. Biol. Chem.*, **234**, 469 (1959).
121. Li, T.-K., Ulmer, D. D. and Vallee, B., *Biochem.*, **2**, 482 (1963).
122. Dixon, M. and Webb, E. C., "Enzymes", Academic Press, New York, 1958, p. 205.
123. Moffitt, W., *J. Chem. Phys.*, **25**, 467 (1956), *Proc. Nat. Acad. Sci., US*, **42**, 736 (1956).
124. Yang, J. T. and Doty, P., *J. Am. Chem. Soc.*, **79**, 761 (1957).
125. Moffitt, W. J. and Yang, J. T., *Proc. Nat. Acad. Sci., US*, **42**, 596 (1956).
126. Shechter, E. and Blout, E. R., *ibid.*, **51**, 695, 794 (1964).
127. Shechter, E., Carver, J. P. and Blout, E. R., *ibid.*, **51**, 1029 (1964).
128. Dorsey, N. E., "Properties of Ordinary Water Substance", ACS Monograph Series, Reinhold Publ. Corp., New York, 1940, p. 287.
129. Tanford, C., Buckley III, C. E., De, P. K. and Lively, E. P., *J. Biol. Chem.*, **237**, 1168 (1962).
130. Havsteen, B. H., *J. Theor. Biol.*, **10**, 1 (1966).

131. Tanford, C., *J. Am. Chem. Soc.*, **84**, 1747 (1962).
132. Litman, B. J. and Schellman, J. A., *J. Phys. Chem.*, **69**, 978 (1965).
133. Davies, D. R., *J. Mol. Biol.*, **9**, 605 (1964).
134. Harris, J. I. and Perham, R. N., *Biochem. J.*, **89**, 60p (1963).
135. Chance, B. and Devault, D., *Ber. Bunsenges. physik. Chem.*, **68**, 722 (1964).
136. Sutin, N. and Christman, D. R., *J. Am. Chem. Soc.*, **83**, 1773 (1961).
137. Keilin, D. and Hartree, E. F., *Proc. Roy. Soc. (London)*, **122 B**, 298 (1937).
138. Tsou, C. L., *Biochem. J.*, **49**, 362 (1951).
139. Theorell, H. and Åkesson, Å., *J. Am. Chem. Soc.*, **63**, 1818 (1941).
140. Russell, C. D. and Pauling, L., *Proc. Nat. Acad. Sci., US*, **25**, 517 (1939).
141. Rodkey, F. L. and Ball, E. G., *J. Biol. Chem.*, **182**, 17 (1950).
142. Hodgman, C. D., (ed.), "Handbook of Chemistry and Physics", 35th ed., Chemical Rubber Co., Cleveland, 1953, p. 1631.
143. Djerassi, C., "Optical Rotatory Dispersion", McGraw Hill, New York, (1960).
144. Imahori, K., *Biochim. Biophys. Acta*, **37**, 336 (1960).
145. Wada, A., Tsuboi, M. and Konishi, E., *J. Phys. Chem.*, **65**, 1119 (1961).
146. Fasman, G. D., Idelson, M. and Blout E. R., *J. Am. Chem. Soc.*, **83**, 709 (1961).
147. Rosenheck, K. and Doty, P., *Proc. Nat. Acad. Sci., US*, **47**, 1775 (1961).
148. Goodman, M., Schmitt, E. E. and Yphantis, D., *J. Am. Chem. Soc.*, **82**, 3483 (1960).
149. Yapel, A., Han, M., Lumry, R., Rosenberg, A. and Shiao, D. F., *J. Am. Chem. Soc.*, **88**, 2573 (1966).
150. Wootton, J. F., Ph. D. dissertation, Cornell University (1960).
151. Eigen, M., and DeMaeyer, L., in "Rapid Mixing and Sampling Techniques in Biochemistry", p. 175, B. Chance, K. H. Eisenhardt, Q. H. Gibson and K. K. Lonberg-Holm, eds., Academic Press, New York, 1964.
152. Erman, J. E. and Hammes, G. G., *Rev. Scient. Instr.*, **37**, 746 (1966).
153. Chance, B., Schoener, B. and Elsaesser, S., *J. Biol. Chem.*, **240**, 3170 (1965).
154. Chance, B., Hess, B. and Betz, A., *Biochem. Biophys. Res. Comm.* **16**, 182 (1964).
155. Chance, B. and Schoener, B., *ibid.*, **17**, 416 (1964).
156. Chance, B., and Roughton, F. J. W. in "Rapid Mixing and Sampling Techniques in Biochemistry", p. 3, 5.
157. Gibson, Q. H. and Milnes, L., *Biochem. J.*, **91**, 161 (1964).
158. Strittmatter, P., in "Rapid Mixing and Sampling Techniques in Biochemistry", p. 71., B. Chance et al., eds. Academic Press, New York, 1964.
159. Diebler, H., Eigen, M. and Matthies, P., *Z. für Naturforschung*, **16 b**, 629 (1961).
160. Meixner, J., *Kolloid Z.*, **134**, 3 (1953).
161. Jost, W., *Z. für Naturforschung*, **2 a**, 159 (1947).
162. Eigen, M., and Matthies, P., *Chem. Ber.*, **94**, 3309 (1961).
163. Matthies, P., Ph. D. dissertation, Göttingen, 1961.
164. Kunitz, M. and Northrop, J. H., *J. Gen. Physiol.*, **18**, 448 (1934).
165. Eisenberg, M. A., and Schwert, G. W., *ibid.*, **34**, 583 (1951).
166. Schellman, J. A., *Compt. Rend. Lab., Carlsberg (Ser. Chim.)*, **30**, 450 (1958).
167. Brandts, J. and Lumry, R., *J. Am. Chem. Soc.*, **83**, 4290 (1961).
168. Wilcox, P. E., *Fed. Proc.*, **16**, 270 (1957).
169. Sarkar, P. K. and Doty, P., *Proc. Nat. Acad. Sci., US*, **55**, 981 (1966).
170. Benesch, R. and Benesch, R., eds., "Sulfur in Proteins", p. 84, 431, 439, 441. Academic Press, New York, 1959.

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